(2)

USAFSAM-TR-90-18

INTERACTION OF IONIZING RADIATION, GENETICALLY ACTIVE CHEMICALS, AND RADIOFREQUENCY RADIATION IN HUMAN AND RODENT CELLS

Martin L. Meltz, Ph.D.
Patricia K. Holahan, Ph.D.
Steven T. Smith, Ph.D.
James J. Kerbacher, Ph.D.
Victor Ciaravino, Ph.D.

Department of Radiology
University of Texas Health Science Center
7703 Floyd Curl Drive
San Antonio, TX 78284-7800

December 1990

Final Report for Period October 1987 - September 1989

Prepared for
USAF SCHOOL OF AEROSPACE MEDICINE
Human Systems Division (AFSC)
Brooks Air Force Base, TX 78235-5301

NOTICES

This final report was submitted by The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas, under contract F33615-87-C-0610, job order 2312-W1-18, with the USAF School of Aerospace Medicine, Human Systems Division, AFSC, Brooks Air Force Base, Texas. Dr. Johnathan L. Kiel (USAFSAM/RZP) was the Laboratory Project Scientist-in-Charge.

When Government drawings, specifications, or other data are used for any purpose other than in connection with a definitely Government-related procurement, the United States Government incurs no responsibility or any obligation whatsoever. The fact that the Government may have formulated or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication, or otherwise in any manner construed, as licensing the holder or any other person or corporation; or as conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

Johnathan L. Kiel

JOHNATHAN L. KIEL, Ph.D.
Project Scientist

David N. Erwin

DAVID N. ERWIN, Ph.D.
Supervisor

George Schwender MD

GEORGE E. SCHWENDER, Colonel, USAF, MC, CFS
Commander

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S) USAFSAM-TR-90-18
6a. NAME OF PERFORMING ORGANIZATION University of Texas Health Science Center 6b. OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION USAF School of Aerospace Medicine (RZP)
6c. ADDRESS (City, State, and ZIP Code) 7703 Floyd Curl Drive San Antonio, TX 78284-7800	7b ADDRESS (City, State, and ZIP Code) Human Systems Division (AFSC) Brooks Air Force Base, TX 78235-5301
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F33615-87-C-0610
8c. ADDRESS (City, State, and ZIP Code)	10 SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO	PROJECT NO	TASK NO	WORK UNIT ACCESSION NO
61102F	2312	W1	18

11. TITLE (Include Security Classification)
Interaction of Ionizing Radiation, Genetically Active Chemicals, and Radiofrequency Radiation in Human and Rodent Cells

12 PERSONAL AUTHOR(S)
Meltz, Martin L.; Holahan Patricia K.; Smith, Steven T.; Kerbacher, James J.; Ciaravino, Victor

13a. TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM 87/10/1 TO 89/9/30	1990, December	79

16. SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	07		Cell Biology; Radiofrequency Radiation; Ionizing Radiation; Genotoxins
20	14		

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The purpose of this project was to investigate the possible interaction between radiofrequency radiation (RFR) and agents which are known to damage DNA. Experiments were performed using exposures of CHO cells to 350, 850, 1200, and 2450 MHz RFR at up to 40 W/kg and temperatures ranging from 37 to 40°C. No genotoxic effect was observed by sister chromotid exchange induction, chromosome aberration induction, or gene mutation (at the thymidine kinase locus). At levels at or below 10 mW/cm^2 and specific absorption rates (SARs) at or below 4 W/kg, there was no evidence that DNA repair was induced or repair of preexisting DNA damage was inhibited. Adriamycin but not mitomycin C caused a statistically significant increase in the frequency of aberrant cells at 40°C with or without RFR. These observations support thermal mechanisms of RFR interaction.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Johnathan L. Kiel	(512) 536-3583	USAFSAM/RZP

DD Form 1473, JUN 86 Previous editions are obsolete

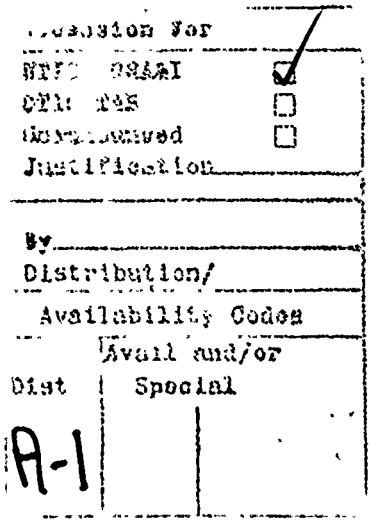
Accession For
NTIS GRA&I
DTIC TAB
Unannounced
Justification
By
Distribution/
Availability Codes
Dist
Avail and/or
Special
A-1

TABLE OF CONTENTS

	Page
INTRODUCTION	1
PRELIMINARY STUDIES	2
Cell Lines	2
I. Objective: To compare the rate of growth of the parent BH4 CHO cell line and its related "mutation sensitive" AS52 CHO cell line	3
II. Objective: To determine the survival response of AS52 cells after treatment with ethyl methane sulfonate (EMS), a known chemical mutagen (for use as a standard positive chemical control)	3
III. Objective: To determine the mutation response of the AS52 cell line to EMS (in our laboratory). EMS is a known mutagen, which was to be used as a positive chemical control in future RFR experiments	6
IV. Objective: To determine the comparative survival response of the AS52 cell line and the parent BH4 cell line after γ-ray exposure from a cesium-137 source	7
Protocol A: Seed cells for cloning efficiency assay 4 hrs before irradiation	7
Protocol B: Survival measurement using the standard chemical mutagen treatment protocol: Seed cells and allow them to attach and grow overnight, irradiate, incubate at 37°C for an additional 19-21 hr, and then resuspend and seed for cloning efficiency assay	11
V. Objective: To determine the mutagenic response of AS52 cells (assayed in our laboratory) after cesium-137 γ-ray exposure	13
VI. Objective: To develop an agar-wall method for exposing mammalian cells (in vitro) simultaneously to ionizing radiation and microwaves, when an external beam source of ionizing radiation is not available	16
VII. Objective: To determine the survival of AS52 cells after ^{32}P β^-exposure for 2 or 4.6 hr (37°C, 50 μCi/ml = 1 mCi/flask)	20
VIII. Objective: To determine AS52 cell survival and mutagenicity as a result of ^{32}P exposure in the agar well system, when the radioactivity concentration was increased to 75 μCi/ml, and one of the incubation times was increased to approximately 6 hr	23

TABLE OF CONTENTS (Continued) Page

IX. Objective: To determine the heat sensitivity of CHO and AS52 cells..... 24

X. Objective: To determine the viability of AS52 cells after heat treatment in agar wells..... 26

XI. Objective: To adapt the propidium iodide staining technique for FACS 420 flow cytometry analysis of the cell types employed in this study..... 31

XII. Objective: To use the FACS 420 flow cytometry system for analysis of effects of selected heat treatments (temperature and time) on the cell cycle distribution of mammalian cells..... 33

COMPLETED STUDIES..... 35

1. Genotoxic Activity of RFR Alone..... 35

2. Alteration of Genotoxic Activity of Chemicals by Simultaneous RFR Exposure to Pulsed Wave RFR at 2450 MHz..... 42

3. Hyperthermic Potentiation of SCE Induction by Adriaycin in CHO Cells..... 47

CONCLUSIONS..... 49

BIBLIOGRAPHY..... 51

APPENDIX A:

Hyperthermic Potentiation of SCE Induction and Cell Killing by Adriamycin in CHO Cells..... 53

GLOSSARY..... 71

LIST OF FIGURES

Figure No.		Page
1	Comparison of the growth rates of the AS52 Chinese Hamster ovary cell line and its parent BH4 cell line.....	4
2	Surviving fraction of AS52 cells after overnight attachment (70% confluent at time of treatment), 5 hr EMS treatment at 37°C, immediate resuspension, and seeding for survival determination..	5
3	Surviving fraction and fraction of clonable cells after EMS treatment of AS52 cells in a mutation experiment.....	8
4	Induced mutants per 1 x 10^6 clonable cells ± S.E., after EMS treatment at various concentrations. The 6-thioguanine selection was initiated on Day 7 post-treatment.....	10
5	(Upper panel): Comparison of the survival response of the AS52 and parent BH4 cell lines, when seeded for cloning efficiency assay 4 hr prior to gamma ray (Cs-137) exposure. (Lower panel): Comparison of the survival response of the AS52 and parent BH4 cell lines, when they are seeded at moderate cell density on the day prior to irradiation, irradiated, and then allowed to incubate overnight before detachment and seeding for viability (Day 1 surviving fraction assay (as in a standard chemical mutagenesis assay)....	12
6	(Upper panel): Comparison of the survival response of the AS52 cells, measured using the two different protocols described in Figure 5. (Lower panel): Comparison of the survival response of BH4 cells, measured using the two different protocols described in Figure 5.....	14
7	Surviving fraction (Day 1) and fraction of clonable cells (Day 7) of AS52 cells after exposure to cesium-137 γ-rays. Data from Expt. 2.....	17
8	Induced mutation frequency per million clonable cells after irradiation of AS52 cells with increasing doses of cesium-137 γ-rays (Expt. 2).....	18
9	Survival of CHO cells, heated at 42.0 - 45.5°C for varying times.	27
10	Survival of CHO cells, heated at 43.0 - 45.5°C for varying times.	28
11	Survival of AS52 cells, heated at 41.0 - 42.5°C for varying times.	29
12	Comparison of survival of CHO and AS52 cells after heating at 42.0 and 42.5°C.....	30
13	FACS 420 Analysis after propidium iodide staining of CHO cells (upper panel) and human lymphoblastoid 244B cells (lower panel).	34
14	Temperature profile in flask during 2-hr exposure.....	37

LIST OF TABLES

Table No.		Page
1	Summary of Initial Surviving Fraction, Fraction of Clonable Cells, and Mutagenicity, for EMS Treated AS52 Cells (5 hr, 37°C).	9
2	Summary of Initial Surviving Fraction, Fraction of Clonable Cells, and Mutagenicity, for Cesium-137 γ-ray Exposed AS52 Cells - Expt. 1 (The cells from 2 independent exposure flasks per dose were combined).....	15
3	Summary of Initial Surviving Fraction, Fraction of Clonable Cells, and Mutagenicity, for Cesium-137 γ-ray Exposed AS52 Cells - Expt. 2 (The data is separately summarized for 4 independent exposure flasks at each exposure dose).....	19
4	Summary of Initial Surviving Fraction for ^{32}P β^-exposure of AS52 Cells for 2 or 4.6 hr (37°C, 50 μCi/ml = 1 mCi/flask).....	22
5	Summary of Initial Surviving Fraction, Fraction of Clonable Cells, and Mutagenicity, for ^{32}P β^-exposure of AS52 Cells for 3.5 or 6.8 hr (37°C, 75 μCi/ml = 1.5 mCi/flask).....	25
6	Summary of Heat Treatment of AS52 Cells for 6 Hours in Agar Wells.....	32
7	Absence of RFR Induction of DNA Repair in Human MR C-5 Cells...	40
8	Absence of Induction of SCEs by Pulsed Wave RFR at Three Frequencies.....	41
9	Absence of Induction of Chromosome Aberrations by 850 MHz PW RFR.....	43
10	Absence of Induction of Chromosome Aberrations by 2450 MHz PW RFR.....	45
11	Absence of Mutation Induction in L5178Y Cells: Exposure to 2450 MHz PW RFR.....	46
12	The Frequency of SCEs in Chinese Hamster Ovary Cells After Exposure to 2450 MHz Radiofrequency Radiation With and Without Adriamycin at Concentrations of 7.75×10^{-7} and 1×10^{-6} M.....	48

INTERACTION OF IONIZING RADIATION, GENETICALLY ACTIVE CHEMICALS, AND RADIOFREQUENCY RADIATION IN HUMAN AND RODENT CELLS

INTRODUCTION

The purpose of this project was to investigate the possible interaction between radiofrequency radiation (RFR) and agents which are known to be damaging to DNA, the genetic material in mammalian cells. Such agents include a wide variety of chemicals, as well as ionizing radiation and ultraviolet light. Several types of chemicals may interact with DNA, including chemotherapeutic drugs (e.g., Adriamycin) and occupationally used chemicals (e.g., hydrazine). The research summarized in this report includes: a) methodological studies, b) preliminary investigations, and c) completed studies. These investigations were conducted to enable us to examine the potential genotoxic effects of RFR (in the microwave range) alone, as well as its possible interaction with selected genotoxic chemicals or ionizing radiation.

Microwave radiation has long been known to elevate temperature and induce hyperthermia, both in vitro and in vivo. In in vitro investigations of RFR effects on mammalian cells, it is essential to measure the temperature of the incubation medium, and, presumably, of the cells therein. The heating which can occur will depend on the average power transmitted to the location of the exposed sample. The temperature achieved will depend on a number of variables, including: 1) flask orientation with respect to the orientation of the antenna horn, 2) amount and depth of medium in the flasks, 3) any movement of the flasks in the field, and 4) any medium (e.g., water or air) flowing around the flasks to dissipate heat (Meltz et al., 1989).

There has been considerable controversy as to whether there are possible nonthermal effects of microwaves at the cytotoxic and genotoxic level. To carefully distinguish between these possibilities, the response of any system to hyperthermia alone must always be accurately measured and analyzed. An important component of these studies was therefore to measure the effect of precisely controlled hyperthermic temperatures (41 - 45°C), to which cells were exposed for varying periods of time, on cell growth and viability (measured either by dye exclusion or colony-forming ability). Since thermal dose is dependent on time at a given temperature as well as the temperature, several different exposure regimens were used.

The genotoxic assays of relevance to this project included the measurement of sister chromatid exchange (SCE) induction in human lymphoblastoid cells (the 244B cell line) and mutation induction in Chinese hamster ovary cells (the AS52 cell line). Because these cytogenetic assays require additional incubation at 37°C after exposure, as part of their standardized experimental assay manipulations, it was also important to assess heat effects at varying times after heating. These hyperthermia studies were performed using convection heating by immersion of the treatment flasks into precision temperature-controlled water baths.

The RFR exposure facility at the U.S. Air Force School of Aerospace Medicine (USAFSAM), where the microwave experiments were carried out, did not have an X-ray source available for simultaneous exposures. Furthermore, a newly

constructed facility, the Center for Basic Research in Radiation Bioeffects (CBRRB), at the University of Texas Health Science Center at San Antonio (UTHSCSA), where simultaneous microwave and X-ray exposures could be performed, was not yet completed. Therefore, a system in which ^{32}P-containing agar was layered into treatment flasks, before the addition of a cell suspension, was developed. This system would allow for simultaneous exposure of cells to β^- particles and microwave radiation. Preliminary experiments were conducted to determine the cytotoxicity and mutagenesis in AS52 cells exposed in this system to β^- radiation.

Preliminary flow cytometry studies were also initiated using a recently acquired FACS 420 flow cytometer. This allowed adaptation of protocols described in the literature for analysis of DNA distribution in both 244B lymphoblastoid cells and CHO cells. These studies will be applied to subsequent investigations.

A complete listing of the project elements in each category appears in the Table of Contents. The Conclusions section of this report summarizes the significant conclusions for all of the material appearing in the report.

A. PRELIMINARY STUDIES

Cell Lines

CHO Cells

Normal (wild-type) Chinese hamster ovary (CHO) cells were obtained from Dr. W.C. Dewey, University of California at San Francisco. This cell line has been well characterized for its response to hyperthermia. These cells were maintained in either 75 cm^2 (T-75) or 25 cm^2 (T-25) tissue culture flasks in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and gentamycin (40 μg/ml). Exponentially growing cultures of cells were incubated at 37°C in a 95% air/5% CO_2 incubator. Cells were passaged 3 times weekly by resuspension using trypsin (0.25% v/v), counting electronically with a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL), and plating at a lower cell density.

AS52 Cells

A transformed line of CHO cells was obtained from Dr. Abe Hsie, University of Texas Medical Branch at Galveston. This cell line was transformed using a bacterial plasmid and is sensitive to ionizing radiation induced mutagenesis at the xanthine-guanine phosphoribosyl transferase (XPRT) gene locus, with no alteration in its normal radiation response for lethality. These cells were maintained in Ham's F12 medium supplemented with 10% HI-FBS and gentamycin (40 μg/ml). Exponentially growing cultures of cells were maintained as described for the CHO cells.

244B Lymphoblastoid Cells

A transformed normal human B lymphocyte cell line (244B) was donated by Dr. J.L. Schwartz (University of Chicago). This lymphoblastoid cell line was maintained in exponential growth in RPMI-1640 medium supplemented with 20% fetal bovine serum, 1 mM α-ketoglutarate, 3 mM L-glutamine, 0.5 mg/ml gentamycin, and 60 units/ml nystatin (complete medium). Cells were seeded at 5 x 10^5 cells/ml

into 75 cm^2 tissue culture flasks and passaged every 48 hr by counting and dilution.

I. Objective: To compare the rate of growth of the parent BH4 CHO cell line and its related "mutation sensitive" AS52 CHO cell line.

Hypothesis: The growth kinetics of the 2 cell lines are the same.

Method of growth curve determination:

Twenty T-25 flasks were seeded with 2 x 10^4 cells in Ham's F12 growth medium supplemented with 10% HI-FBS. The flasks were placed in a humidified 5% CO_2/95% air incubator (loose caps) and incubated for different times at 37°C. At each time interval selected, 2 flasks were removed from the incubator. The cells were detached using a standard trypsination technique and resuspended in a small volume of fresh medium. The cell density was determined using an electronic counter (Model ZBI Coulter Counter), and the total cell number per flask calculated for the volume of suspended cells in each flask. The average values of 2 flasks per time point are shown for the 2 cell lines in Figure 1.

Result: The 2 growth curves were nearly identical, with a population doubling time of 13.5 hr for the parent BH4 cells, and 14.5 hr for AS52 cells.

II. Objective: To determine the survival response of AS52 cells after treatment with ethyl methane sulfonate (EMS), a known chemical mutagen (for use as a standard positive chemical control).

Hypothesis: The survival of AS52 cells would be decreased by treatment with ethyl methane sulfonate (EMS) in the concentration range 50 - 2400 μg/ml.

Method of treatment

One day prior to treatment, cells were seeded into 16 T-25 flasks at 5 x 10^5 cells per flask in 5 ml of Ham's F12 medium with 5% HI-FBS. After dilutions of the high concentration EMS stock solution were made into sterile saline, so that when 0.1 ml aliquots were added to the flasks, final concentrations of 1200, 900, 600, 300, 100 and 50 μg/ml resulted. After the 5-hr incubation, the cells were washed 3 times with Saline G. They were then detached using trypsin, resuspended in fresh medium, and replicate flasks combined; the cells were then counted electronically. For each treatment condition at the lower chemical concentrations, cells were seeded into 8 T-25 flasks for cloning efficiency determination (and survival calculation); they were seeded into 10 flasks for the 1200 and 2400 μg/ml treatment concentrations. The flasks were incubated for 7 or 8 days, stained with crystal violet, and the number of colonies containing more than 50 cells counted. Survival was corrected for control plating efficiency.

Results

The results of the experiment are plotted in Figure 2. It can be seen that, for this lot of EMS, the concentration of 300 μg/ml did not result in measurable cell killing. At 600 μg/ml, the surviving fraction was 0.84, and

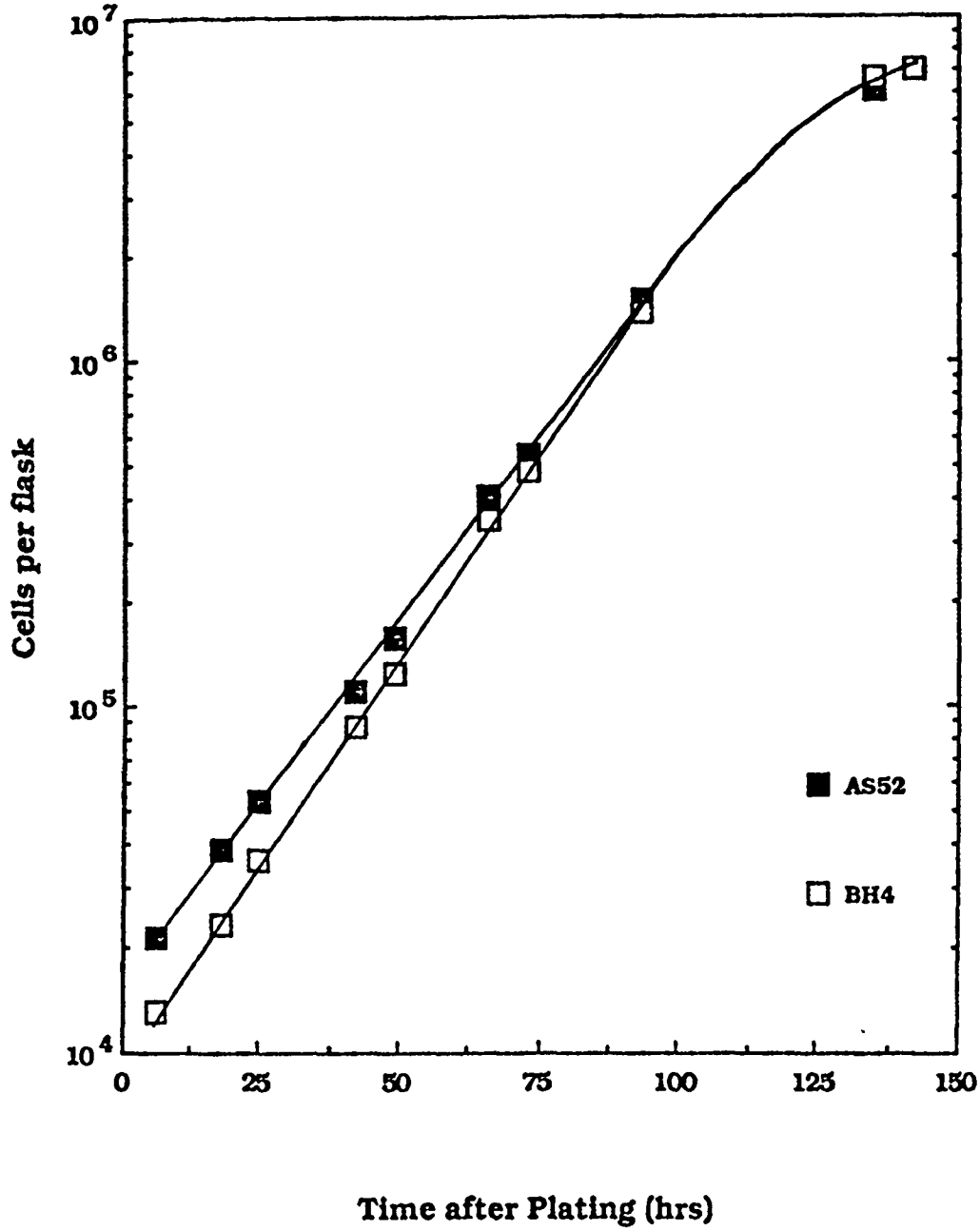

Figure 1. Comparison of the growth rates of the AS52 Chinese Hamster ovary cell line and its parent BH4 cell line.

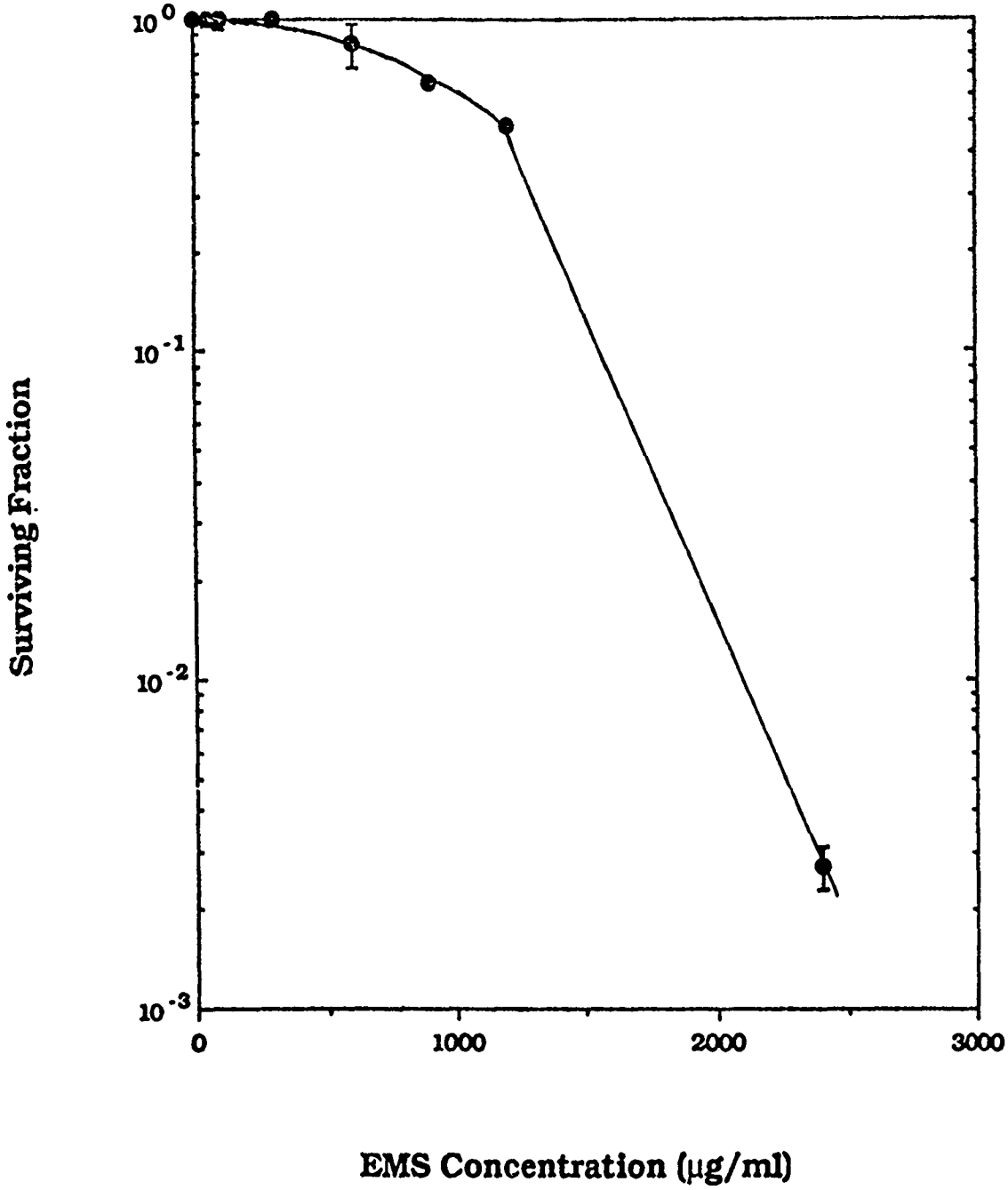

Figure 2. Surviving fraction of AS52 cells after overnight attachment (70% confluent at time of treatment), 5-hr EMS treatment at 37°C, immediate resuspension, and seeding for survival determination.

at 1200 μg/ml it was 0.49. At the concentration of 2400 μg/ml, the surviving fraction had been reduced to 3×10^{-3}.

III. Objective: To determine the mutation response of the AS52 cell line to EMS (in our laboratory). EMS is a known mutagen, which was to be used as a positive chemical control in future RFR experiments.

Hypothesis: Treatment with increasing concentrations of EMS will result in an increasing mutation frequency.

Methods

In the week prior to the mutation experiment, cells were incubated for 2 days in MPA medium to "purify" the population of preexisting mutant (6-thioguanine resistant) cells. The cells were then detached and seeded into 3 flasks in fresh complete F12CM5 medium so that approximately 8×10^6 cells would be available on the following Monday. At that time, an inoculum of 5×10^5 cells per flask was seeded into 20 T-25 flasks with F12CM5 with dialyzed serum (F12CM5-DS). On the following day, the attachment medium was removed, and replaced with 4.9 ml of fresh F12CM5-DS medium. The flasks were incubated for 1 hr at 37°C. To each flask, 0.1 ml of EMS from the appropriate stock dilution was then added. Four flasks were treated at each concentration. The flasks were incubated for 5 hr at 37°C. After the 5-hr incubation, the treatment medium was removed by pipet, and each flask was rinsed 3 times with Pucks Saline G. To each flask, 5 ml of fresh, warm F12CM5 was added, and the flasks were incubated overnight (to recover from treatment). The cells in each flask were then detached using trypsin, and resuspended in fresh medium. The cells were counted (Day 1) and diluted with F12CM5-DS. For measurement of the initial surviving fraction, 200 cells were seeded in 3 flasks. These were incubated for 8 days, and then stained, and the colonies were counted. To allow for expression (as part of the mutation assay), for each of the initial treatment flasks, 1×10^6 cells were seeded into T-75 flasks in 18 ml of F12CM5-DS medium. These flasks were incubated at 37°C. On Days 3 and 5, the cells in each flask were again resuspended and counted, and then seeded in fresh T-75 flasks at 1×10^6 cells per flask. On Day 7, after resuspension, 2×10^5 cells from each treatment were seeded into each of 5 T-75 flasks (10^6 cells total were seeded from each treatment flask) in hypoxanthine-free F12FCS5-DS medium containing 6-thioguanine at a final concentration of 10 μM. These flasks for mutation selection were then incubated for 8 days. In addition, 200 cells from each final resuspension were seeded into each of 5 T-25 flasks in F12FCS5-DS medium (without the selection agent). These flasks, for the assay of the fraction of clonable cells, also were incubated for 8 days, at which time all of the flasks were stained and counted.

The number of stained colonies in the 6-thioguanine containing flasks were determined, as was the cloning efficiency in the Day 7 fraction of clonable cell flasks. The mutation frequency was then calculated from this data, using the formula

$$\frac{\text{number of 6-thioguanine resistant colonies (in the 5 dishes)}}{1 \times 10^6 \times \text{cloning efficiency of Day 7 "fraction of clonable cell" plates}}$$

Results

In this experiment, the cells were treated with final EMS concentrations of 0, 100, 300, 600, and 1000 μg/ml. Four flasks were treated at each concentration. For each treatment flask, 4 "initial" cloning efficiency flasks were seeded. After colony counting, the average cloning efficiency of the 4 assay flasks was determined, and then the surviving fraction was calculated for each treatment flask. The average surviving fractions, +/- standard error, were then calculated for the 4 treatment flasks of each chemical concentration. These average values, with their standard errors, are plotted in Figure 3, and represent the surviving fraction resulting from the initial treatment.

As already indicated, final cloning efficiency values were also determined for the cells present in the population at the time (Day 7 posttreatment) that the 6-thioguanine was added to cells for mutant selection; this value was used in the calculation of mutant frequency. In this study, 5 flasks were seeded for each treatment flask. The calculation of average fraction of clonable cell and standard error is as described above. This curve for fraction of clonable cells is also plotted on Figure 3.

The summary of all of the data for EMS treated cells appears in Table 1, and the mutagenicity is summarized in Figure 4.

Discussion

As seen in Figure 3, the surviving fraction after EMS exposure, when measured on Day 1 after treatment, is less than the measured fraction of clonable cells on Day 7. At this later time, the cells have either recovered from the initial treatment, and/or dead cells have been increasingly diluted by growth of the viable cells in the population.

Examination of the data in Table 1 and Figure 4 shows the steady increase in the induced mutation frequency with increasing EMS concentration, both supporting the hypothesis and demonstrating the mutagenicity of EMS in this system in our laboratory.

IV. Objective: To determine the comparative survival response of the AS52 cell line and the parent BH4 cell line after γ-ray exposure from a cesium-137 source.

Protocol A: Seed cells for cloning efficiency assay 4 hr before irradiation.

Hypothesis: The radiation dose survival response of the 2 cell lines would be equivalent using this standard viability assay for reproductive integrity.

Methods

Cells of both cell stocks were resuspended, counted, and seeded into T-25 flasks at varying cell densities to obtain approximately 30-100 colonies per flask after each dose (based on data from other laboratories).

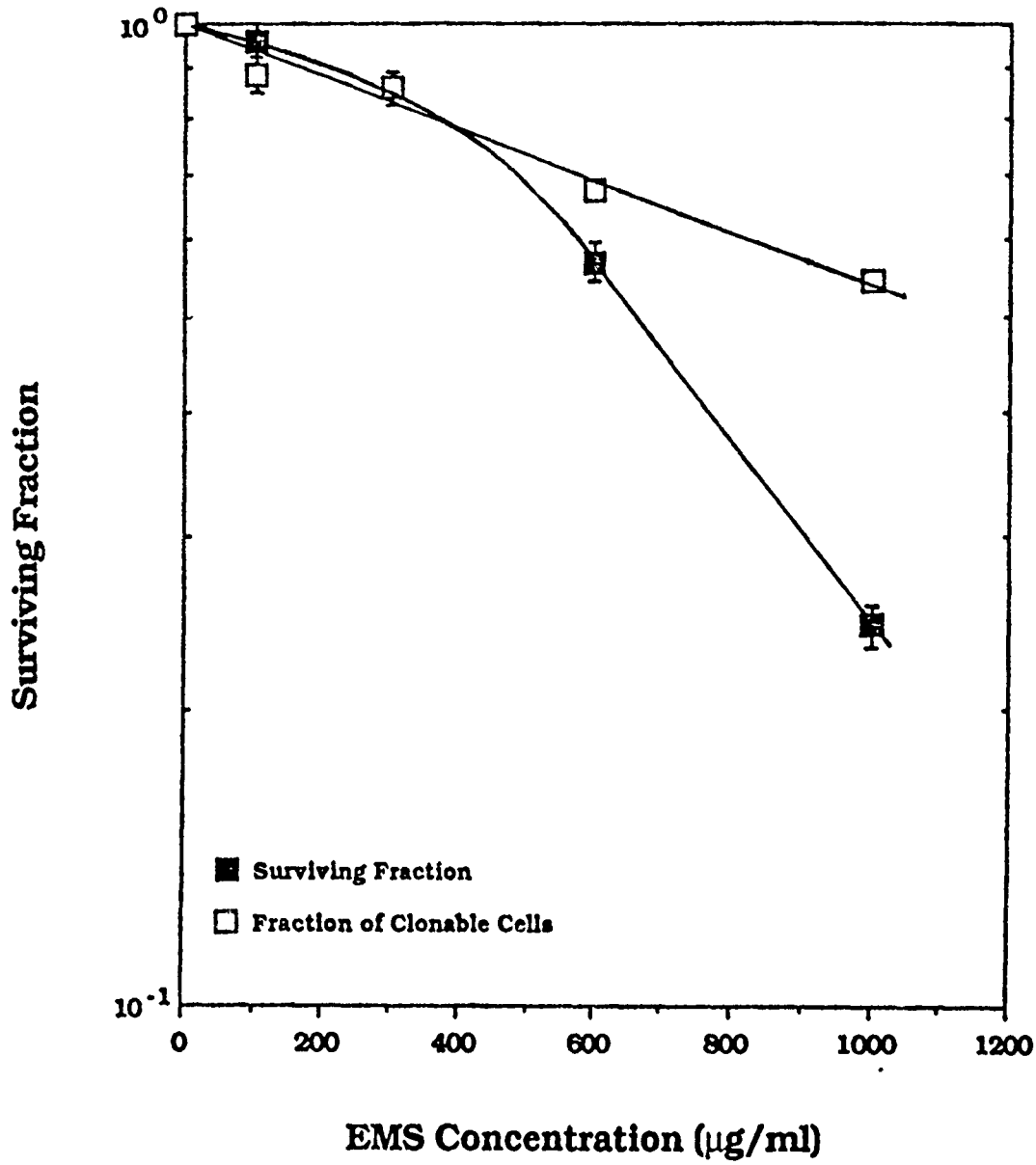

Figure 3. Surviving fraction and fraction of clonable cells after EMS treatment of AS52 cells in a mutation experiment. Open Squares = Initial surviving fraction measured on Day 1 (Posttreatment). Closed Squares = Final fraction of clonable cells measured on Day 7.

TABLE 1. SUMMARY OF SURVIVING FRACTION, FRACTION OF CLONABLE CELLS, AND MUTAGENICITY FOR EMS TREATED AS52 CELLS (5 HR, 37°C)

EMS Concentration (Replicates 1-4)	Initial (Day 1) Cloning Efficiency (Mean ± SE) (n=5)	Initial (Day 1) Surviving Fraction (Mean ± SE) (n=5)	Day 7 Cloning Effic. (Mean ± SE)	Day 7 Fraction of Clonable Cells (Mean ± SE) (n=5)	Mut. Frequency per Clonable Cell ($x10^{-6}$)	Induced Mut. Freq. ($x10^{-6}$)
0 g/ml 1	.825±.026		.948±.043		6.34	
2	.713±.030		.878±.049		10.27	
3	.731±.048		1.047±.022		21.97	
4	.668±.048		.979±.016		19.40	
Mean =	.734±.035		.963±.040		14.50±4.27	
100 µg/ml 1	.523±.018	.713±.024	.884±.049	.852±.034	60.04	45.54
2	.663±.028	.903±.038	.889±.043	.923±.045	89.55	75.05
3	.820±.038	1.117±.052	.918±.037	.953±.038	57.79	43.29
4	.801±.027	1.092±.036	.785±.026	.815±.027	80.15	65.65
Mean =	.701±.080	.956±.109	.869±.033	.886±.037	71.88±8.94	57.38±8.94
300 µg/ml 1	.599±.032	.816±.044	.904±.045	.939±.047	157.29	142.79
2	.613±.022	.835±.029	.780±.032	.810±.033	167.25	152.75
3	.657±.032	.895±.043	.791±.020	.821±.020	249.84	235.34
4	.653±.014	.889±.019	.826±.028	.858±.030	197.14	182.64
Mean =	.631±.017	.859±.021	.825±.032	.857±.034	192.88±24.00	178.38±24.00
600 µg/ml 1	.455±.040	.620±.065	.642±.026	.667±.027	584.41	569.91
2	.437±.025	.595±.034	.677±.021	.703±.022	632.54	618.04
3	.383±.012	.522±.016	.615±.030	.639±.031	720.75	706.25
4	.399±.008	.544±.011	.669±.021	.694±.022	572.94	558.44
Mean =	.419±.019	.570±.026	.651±.016	.676±.017	627.66±38.81	613.16±38.81
1000 µg/ml						
1	.166±.006	.226±.008	.540±.016	.560±.016	1333.26	1318.76
2	.195±.008	.266±.011	.499±.015	.517±.016	922.67	908.17
3	.180±.016	.256±.022	.525±.017	.546±.018	1147.42	1132.92
4	.165±.008	.225±.011	.543±.031	.564±.033	977.76	963.26
Mean =	.177±.008	.243±.012	.527±.012	.547±.012	1095.28±106.96	1080.78±106.96

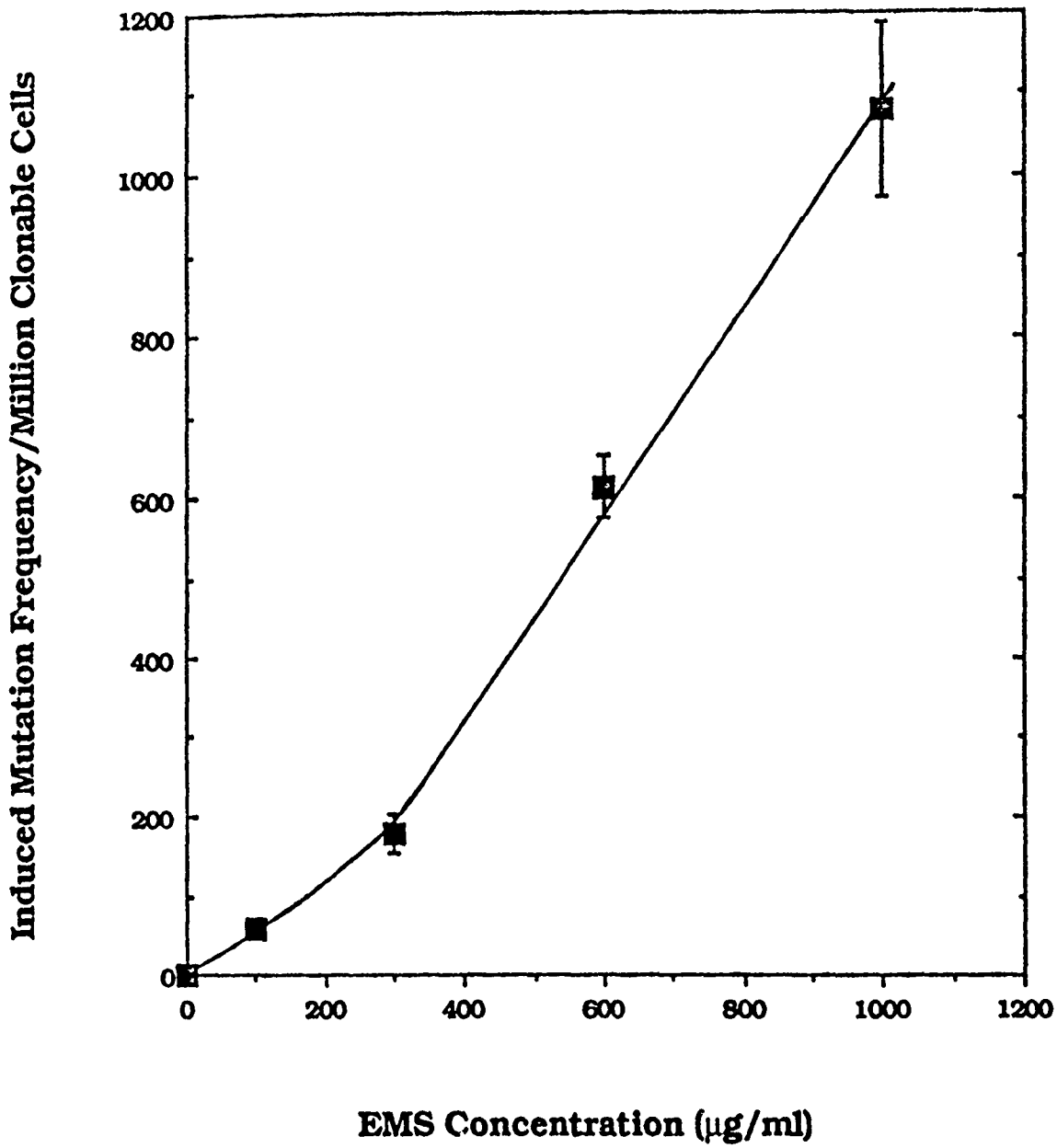

Figure 4. Induced mutants per 1 x 10^6 clonable cells ± S.E., after EMS treatment at various concentrations. The 6-thioguanine selection was initiated on Day 7 posttreatment.

For the lower doses, 8 flasks were seeded at the selected cell density. At doses of 6 Gy and above, 5 flasks were seeded with 1 cell density and 5 flasks were seeded at a higher cell density. Eight flasks were seeded at one very high cell density for the highest dose. After seeding, the flasks were incubated at 37°C in a 5% CO_2/95% air incubator for 4 hr to allow for cell attachment.

The irradiation doses selected were 0, 2, 4, 6, 8, 10, and 12 Gy. The irradiation was performed at room temperature in an Atomic Energy of Canada (AEC) Gamma-Cell 40 Cesium irradiator at a dose rate of 1.2 Gy/min. The flasks were irradiated with a sequence alternating between a low total dose and a high total dose. The flasks were maintained at 37°C until immediately before irradiation of each exposure dose set.

Two multiplicity flasks (for each cell type) were fixed immediately before irradiation began, and 2 were fixed immediately after exposures were completed. Immediately after exposure, the flasks in each set were returned to the humidified incubator and incubated for 8 days until staining.

Results

The average values of the colonies seeded in each irradiation set (8 or 10 flasks) for each dose were determined. The surviving fraction was then calculated relative to the cloning efficiency of the unirradiated control (as 100% survival). The data was corrected for multiplicity. The average surviving fraction data with standard errors are plotted in Figure 5 (upper panel) for Protocol A for the AS52 and BH4 cell lines. From Figure 5 (upper panel) it appears that, using a standard 4-hr attachment interval prior to irradiation, the AS52 cell line is slightly more sensitive to gamma ray killing than the parent BH4 cell line.

Protocol B: Survival measurement using the standard chemical mutagen treatment protocol: Seed cells at moderate cell density and allow them to attach and grow overnight, irradiate, incubate at 37°C for an additional 19-21 hr, and then resuspend and seed for cloning efficiency assay.

Hypothesis: That the percent survival would be significantly higher if the cells were allowed to incubate overnight at 37°C after irradiation and before trypsinization and plating for colony formation.

Methods

For each cell line and exposure dose, 1 T-25 flask was seeded with 5×10^5 cells on the day before irradiation (7 flasks total). The flasks were incubated overnight, and then exposed using the same doses, sequence of doses, and dose rate described previously. Immediately after exposure, the medium was changed (to mimic the case after chemical treatment) and the flasks were returned to the incubator for overnight incubation at 37°C (19-21 hr). On the next day, the cells were resuspended using trypsin, and diluted and plated as for the 4-hr seeding and exposure protocol. They were incubated for 8 days, stained, and counted.

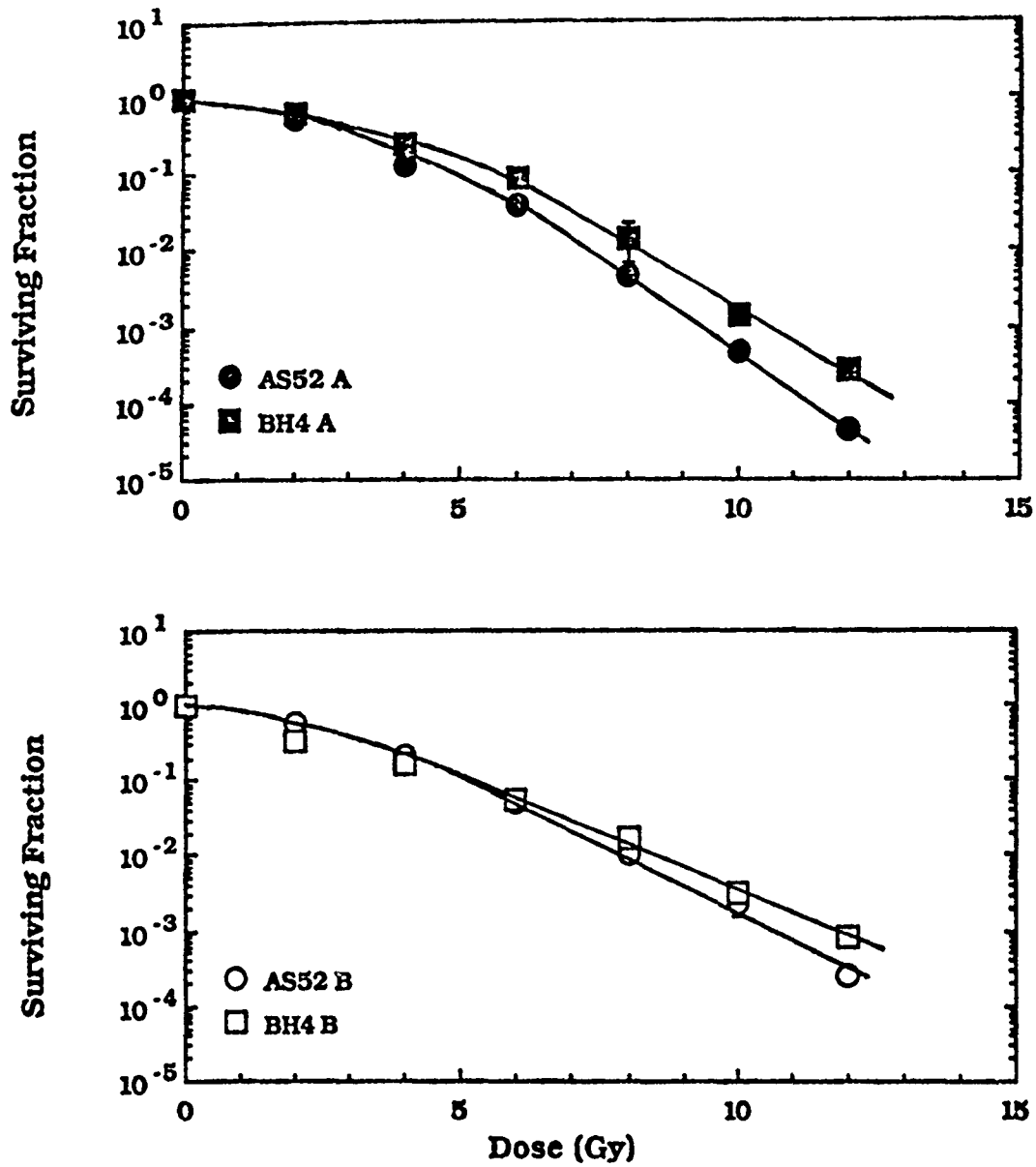

Figure 5. (Upper panel): Comparison of the survival response of the AS52 and parent BH4 cells lines, when seeded for cloning efficiency assay 4 hr prior to gamma ray (Cs-137) exposure.
(Lower panel): Comparison of the survival response of the AS52 and parent BH4 cell lines, when they are seeded at moderate cell density on the day prior to irradiation, irradiated, and then allowed to incubate overnight before detachment and seeding for viability (Day 1 surviving fraction) assay (as in a standard chemical mutagenesis assay).

Results

The results for this irradiation and overnight incubation protocol, for both the BH4 and AS52 cell lines, are shown in the lower panel of Figure 5. Using this protocol, it appears that the sensitivity of the AS52 cells to cesium-137 gamma rays is the same as that of the parent BH4 cell line. In Figure 6, these same data, for the 4-hr vs. overnight protocols, are plotted for the AS52 cells (upper panel), and for the BH4 parent cell line (lower panel). It is evident from these figures that the greatest "contribution" to any difference in gamma ray sensitivity is the result of the lower survival for the AS52 cells determined using the overnight incubation protocol.

V. Objective: To determine the mutagenic response of AS52 cells (assayed in our laboratory) after Cesium 137 γ-ray exposure

Methods

AS52 cells (MPA treated) were seeded at 5 x 10^5 cells/flask into 5 ml F12CM5-DS in 8 T-25 flasks on the day before irradiation. Immediately before exposure, the attachment medium was removed and replaced with fresh, warm F12CM5-DS. The flasks were irradiated with doses of 0, 2, 4, and 6 Gy. The dose rate was 1.19 Gy/min. The flasks were taken from the 37°C incubator immediately before each exposure (at ambient temperature), and they were returned to the 37°C incubator immediately after each exposure and incubated overnight.

On the following morning (19 hr postexposure) the cells were resuspended using trypsin, and counted electronically. For initial surviving fraction determination, the cells were diluted so that 5 ml of medium contained 200 cells. In Experiment 1, the resuspended cells from the 2 flasks irradiated at each dose were combined prior to counting, seeding for initial surviving fraction determination, and subsequent incubation for 72 hr for expression. In Experiment 2 and all other experiments, the independent treatment flasks at each dose were separately assayed. For each dose, 5 flasks were seeded. These were incubated for 8 days; the colonies were then stained and counted. From each of the initial resuspensions (Day 1), inoculations of 1 x 10^6 cells were made into T-75 flasks. These were incubated at 37°C. The cells in each of these flasks were resuspended and rediluted on Days 3 and 5. On Day 7, the cells were resuspended and plated for both fraction of clonable cell assay and mutagenic selection, as described in the standard protocol used for the EMS experiment. Five flasks with 200 cells per flask were seeded for fraction of clonable cell determination, and 5 flasks with 2 x 10^5 cells per flask were seeded for 6-thioguanine (10 μM) selection.

Results

Table 2 summarizes the data for Cs-137 Experiment 1, where the cells from the two independent treatment flasks at each dose were combined. The ability of cesium-137 gamma rays to induce mutation was detectable at 2 Gy, where the initial cell viability was 61%. After an exposure of 4 Gy, an approximate 5-fold increase in mutation frequency was observed (153 x 10^{-6} mutants/clonable cell), compared to the spontaneous mutation rate (30 x 10^{-6} mutants/clonable cell) for the 0 dose control.

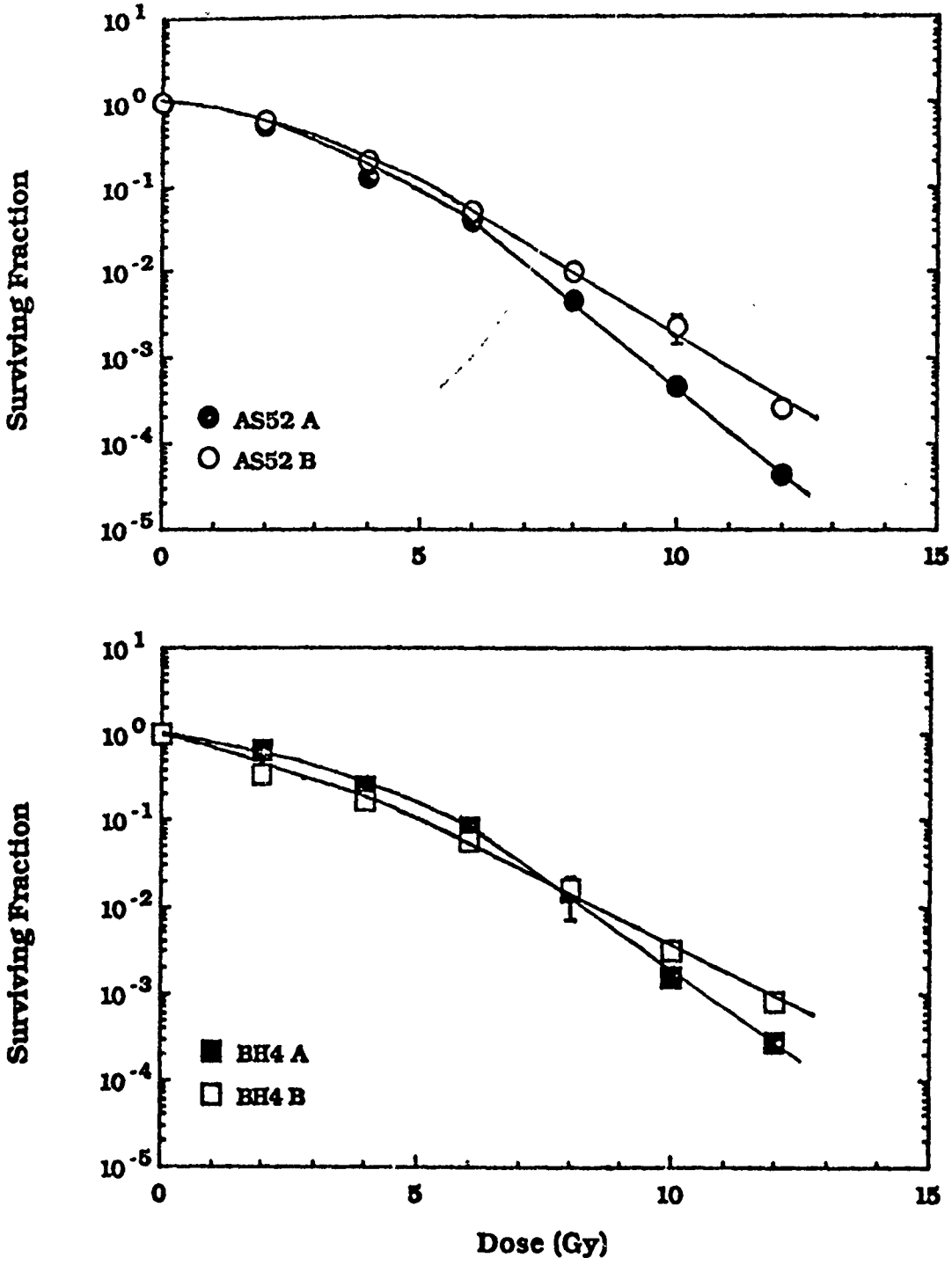

Figure 6. (Upper Panel): Comparison of the survival response of the AS52 cells, measured using the 2 different protocols described in Figure 5. (Lower Panel): Comparison of the survival response of BH4 cells, measured using the 2 different protocols described in Figure 5.

TABLE 2. SUMMARY OF INITIAL SURVIVING FRACTION, FRACTION OF CLONABLE CELLS, AND MUTAGENICITY FOR CESIUM-137 γ-RAY EXPOSED AS52 CELLS - EXPT. 1. (CELLS COMBINED FROM 2 INDEPENDENT EXPOSURE FLASKS PER DOSE)

Exposure Condition	Initial (Day 1) Cloning Effic. (Mean ± SE)	Initial (Day 1) Surviving Fraction (Mean ± SE) (n=5)	Day 7 Cloning Effic. (Mean ± SE)	Day 7 Fraction of Clonable Cells (Mean ± SE) (n=5)	Mut. Frequency per Clonable Cell (x10-6) (± SE)	Induced Mut. Frequency ($x10^{-6}$) (± SE)
0 Gy	.654±.028		.812±.038		29.60	
2 Gy	.398±.007	.608±.011	.856±.031	1.054±.039	57.31	27.71
4 Gy	.113±.006	.172±.010	.776±.004	.956±.005	182.85	153.25
6 Gy	.033±.002	.051±.003	.670±.020	.825±.024	630.18	600.58

The results of a second and more comprehensive experiment to measure the toxicity, fraction of clonable cells, and mutagenicity after exposure of AS52 cells to γ-ray exposure from a cesium-137 source (Experiment 2) are summarized in Figures 7 and 8, and Table 3. As expected, the surviving fraction (Day 1) shows a continuing decrease with increasing exposure dose. Also as expected, the fraction of clonable cells, measured at Day 7, has a much higher value for each exposure dose. The ability of γ-irradiation to induce mutations in this cell line was again detected at 2 Gy (200 rads).

The mean value of the induced mutation frequency at this dose, $69\pm11 \times 10^{-6}$ mutants/clonable cell, is more than double the mutation frequency of the 37°C control ($15\pm5 \times 10^{-6}$ mutants/clonable cell). The extent of induced mutagenicity is observed to increase with increasing dose. The maximum induced mutation frequency observed in this experiment was a mean value of $333\pm67 \times 10^{-6}$ mutants/clonable cell after 6 Gy, lower than observed in the initial experiment. It is interesting to note that at approximately equivalent levels of survival after ionizing radiation and chemical mutagen treatment, i.e., a mean of 67.5% for 2 Gy of γ-ray, and 58.2% after incubation with 1000 μg/ml EMS for 5 hr at 37°C, the respective mean induced mutant frequencies were 69×10^{-6} and 773×10^{-6} induced mutants per clonable cell. The EMS appears to be much more "efficient" as a mutagen, at equally low toxicity levels, in this system.

VI. Objective: To develop an agar well method for exposing mammalian cells (in vitro) simultaneously to ionizing radiation and microwaves, when an external beam source of ionizing radiation is not available.

Methods

We describe here our effort to develop an agar well system in T-25 tissue culture flasks, where the agar bottom layer contains the radioactive isotope ^{32}P. The goal was to establish an exposure geometry where it would "appear" to the cells that the radiation was coming from an infinite source. To accomplish this, it was theorized (by Dr. Jack Lancaster, a consultant to this project) that the agar layer would have to be approximately 1 cm thick, and that the cells would have to be located at least 1 cm away from any edge of the radioactive agar layer.

To create such a geometry, the following "agar well" design in T-25 flasks was developed:

1. Into a T-25 flask, 20 ml of hot sterile 6% BBL agar is added. The flask is capped tightly and turned upside down, so that on cooling, an agar plug forms in the neck opening.

2. The cap is removed, and a sterile needle catheter with stylus (13- or 14-gauge stainless steel) is inserted into the top side of the agar plug; the stylus is then removed. This catheter allows air to vent when more agar is inserted. A second needle catheter with stylus is then inserted, and the latter is removed. A 12-ml syringe (with plunger removed) is attached to the second needle. A 10-ml volume of hot 6% agar, already containing the appropriate amount of radioactivity, is poured into the syringe barrel. The ^{32}P is in the chemical form adenosine 5'-triphosphate, gamma-^{32}P, unpurified (New England Nuclear). The plunger is inserted back into the syringe, and the hot agar is injected into the flask. The flask (behind appropriate shielding) is placed flat on the counter so that the hot agar will settle and harden as a

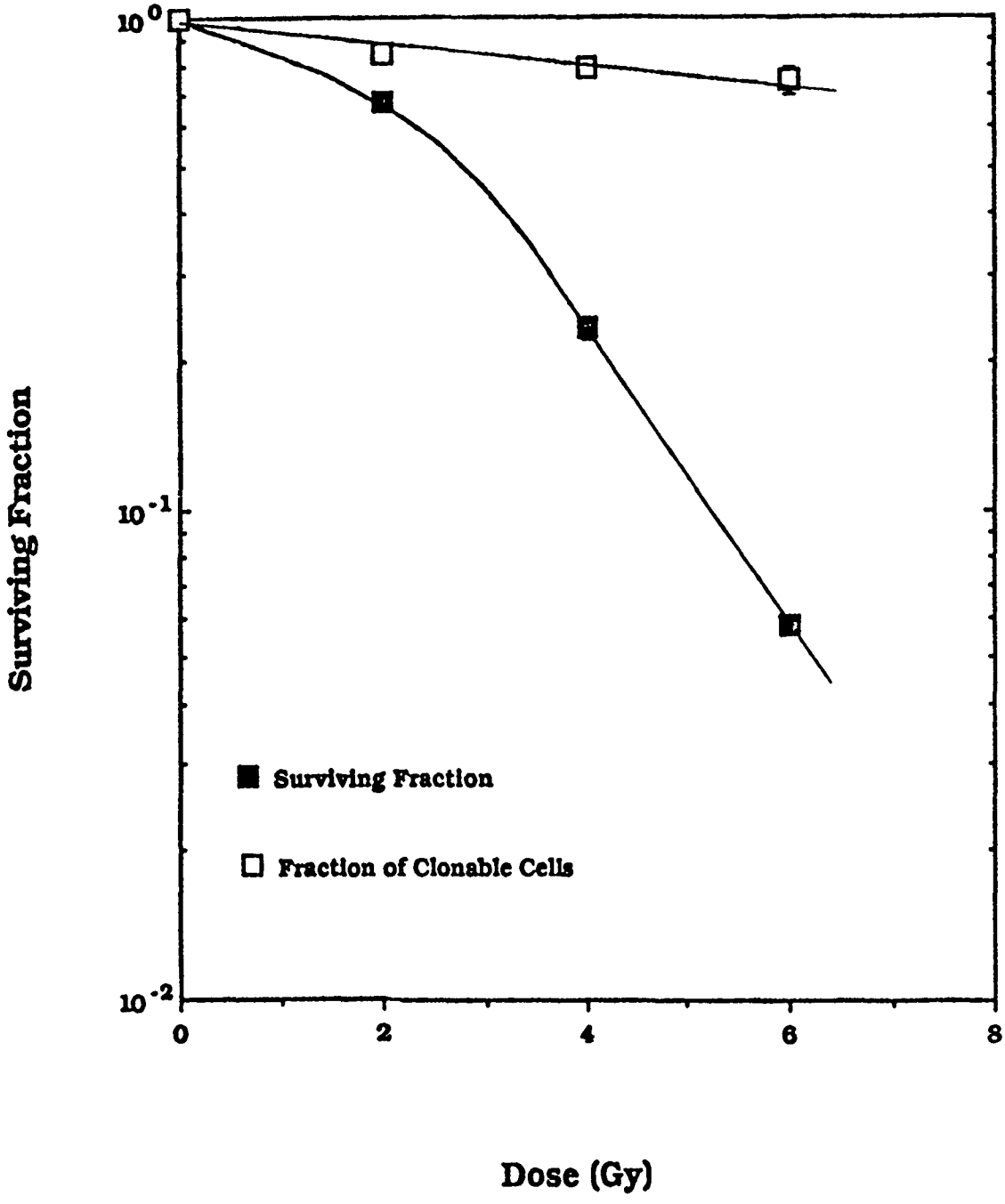

Figure 7. Surviving fraction (Day 1) and fraction of clonable cells (Day 7) of AS52 cells after exposure to cesium-137 γ-rays. Data from Experiment 2. The values plotted are the mean ± SE for 4 independent treatment flasks at each dose.

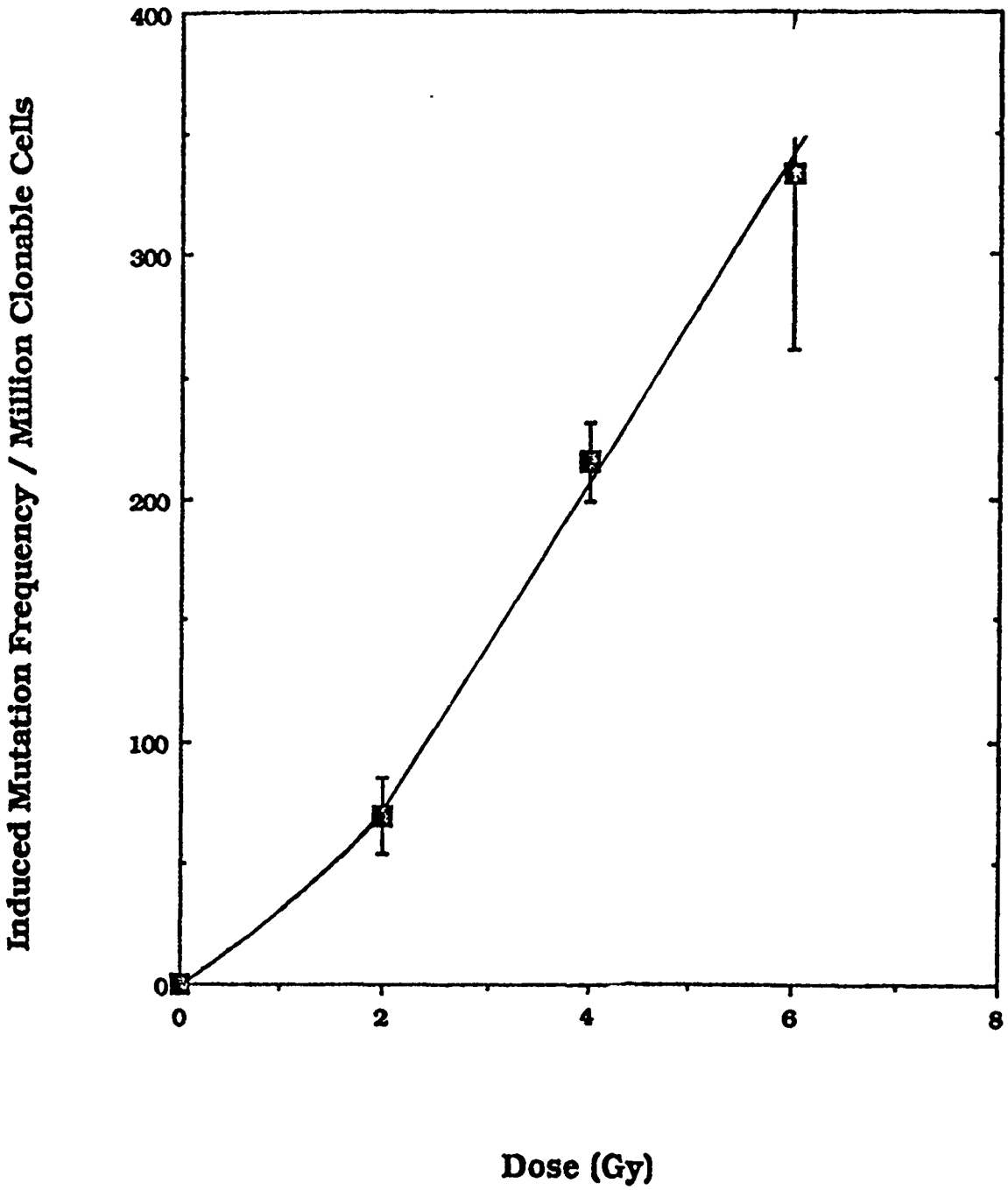

Figure 8. Induced mutation frequency per million clonable cells after irradiation of AS52 cells with increasing doses of cesium-137 γ-rays (Experiment 2). Values plotted are the mean ± SE for 4 independent treatment flasks at each dose.

TABLE 3. SUMMARY OF INITIAL SURVIVING FRACTION, FRACTION OF CLONABLE CELLS, AND MUTAGENICITY, FOR CESIUM-137 γ-RAY EXPOSED AS52 CELLS - Expt. 2

Exposure Condition (Replicates 1-4)		Initial (Day 1) Cloning Effic. (Mean ± SE)	Initial (Day 1) Surviving Fraction (Mean ± SE) (n=5)	Day 7 Cloning Effic. (Mean ± SE)	Day 7 Fraction of Clonable Cells (Mean ± SE) (n=5)	Mut. Frequency per Clonable cell ($x10^{-6}$)	Induced Mut. Freq. ± SE ($x10^{-6}$)
0 Gy	1	.887±.032		.941±.025		28.69	
	2	.845±.021		.924±.033		9.74	
	3	.792±.019		1.031±.039		14.56	
	4	.888±.032		.885±.033		9.05	
Mean =		.853±.026		.945±.036		15.51±5.27	
2 Gy	1	.567±.02	.655±.024	.738±.020	.781±.021	120.56	105.05
	2	.613±.037	.719±.044	.838±.021	.887±.022	66.88	51.37
	3	.612±.028	.717±.033	.746±.023	.790±.024	91.16	75.65
	4	.509±.019	.597±.022	.861±.031	.911±.033	60.49	44.98
Mean =		.575±.028	.675±.033	.796±.036	.842±.038	84.77±15.75	69.26±15.75
4 Gy	1	.209±.014	.245±.016	.767±.042	.812±.045	248.82	233.31
	2	.209±.005	.245±.003	.689±.016	.729±.017	214.60	199.09
	3	.175±.012	.205±.014	.707±.032	.748±.034	199.91	184.40
	4	.202±.026	.237±.031	.824±.015	.872±.016	260.05	244.54
Mean =		.198±.009	.233±.011	.746±.035	.790±.038	230.85±16.32	215.34±16.32
6 Gy	1	.051±.013	.060±.015	.602±.021	.637±.022	509.28	493.77
	2	.050±.007	.059±.008	.830±.044	.864±.035	204.22	188.71
	3	.050±.013	.059±.015	.699±.007	.740±.007	338.86	322.85
	4	.046±.005	.054±.006	.696±.024	.737±.026	343.60	328.09
Mean =		.049±.001	.058±.002	707±.054	.745±.046	348.99±72.10	333.48±72.11
EMS, 1000 μg/ml							
	1	.530±.024	.621±.029	.664±.031	.703±.032	764.14	748.63
	2	.433±.010	.508±.011	.643±.023	.680±.024	857.78	842.27
	3	.550±.031	.593±.025	.594±.013	.629±.014	781.13	765.62
	4	.517±.019	.606±.022	.644±.090	.618±.038	752.17	736.66
Mean =		.508±.03	.582±.029	636±.017	.658±.023	788.81±27.42	773.32±27.42

radioactive layer on the bottom surface of the flask (the surface to which cells would normally attach). This volume of agar results in an agar layer approximately 1 cm thick. Immediately after loading radioactive agar into one flask, the stylus is returned to both the vent and radioactive needle catheters; these are then removed and inserted into and through the agar plug in the next flask to be filled. Once the radioactive agar layer has been added to each of the flasks, that flask is placed into a small Plexiglas holder which had been constructed to hold an individual T-25 flask. This holder reduced the radiation exposure of the scientific personnel during subsequent handling of the flasks.

3. After the radioactive agar bottom layer had hardened, the vent needle catheter and a fresh (nonradioactive) needle catheter (with styli) are inserted through the agar plug at the neck of a flask, and 5 ml of hot 6% agar is added. The flask was turned on its long side edge and allowed to stand until the agar hardened. After doing this for each flask, the process is repeated for the other long side edge.

4. Next, 3 ml of hot 6% agar is added to each flask; the flasks are then allowed to stand upright on their ends to create the third border of the agar well.

5. Because the agar plug was repeatedly penetrated during the previous steps, a final 10-ml volume of hot 6% agar is added using a syringe and needle catheter, and the flask is again turned with its neck and cap in the vertical downward direction. After hardening, this additional agar seals the neck of the flask and also provides the fourth border of the well in the center of the flask; each border keeps the cells at least 1 cm from the edge of the lower ^{32}P agar layer. After the flasks are prepared, they are placed, still in their individual Plexiglas holders, into a Plexiglas box (already at 37°C). They can be refrigerated, but, on the day prior to cell exposure, they are placed into a 37°C incubator. This allows the agar to equilibrate at 37°C before the cells are added for exposure on the next day.

VII. Objective: To determine the survival of AS52 cells after ^{32}P β^-exposure for 2 or 4.6 hr (37°C, 50 μCi/ml = 1 mCi/flask).

Hypothesis: that these exposure conditions will be sufficient to cause AS52 cell survival to decrease to 25% of control after a 5-hr exposure.

Exposure Protocol

Preparation of Agar Layer

Six radioactive agar well flasks were prepared as previously described. On the day prior to the cell exposure, enough radioactivity was added into 120 ml of hot agar so that on the following day, at the pre-selected time of exposure, the radioactivity concentration would be 50 μCi/ml.

Cell Exposure - At the pre-selected time, 8 ml of complete Ham's F12CM10 medium containing a total of 1 x 10^6 AS52 cells was injected into each of the 6 flasks with radioactivity, and also into 3 flasks (similarly prepared) but which lacked any radioactivity ("Control flasks"). In this experiment, the cells were first loaded into the three control flasks. They were then loaded into the 3 flasks for what was planned to be a 4-hr exposure, followed by the 3 flasks for a 2-hr exposure. This sequence proved to be less than desirable, because the

experimental handling of the 2-hr exposed cells overlapped with the desired (and therefore extended) end of the 4-hr incubation. After the cells were injected into each flask, they were capped, and as each set was loaded with cells, the flasks in their Plexiglas holders were placed in the Plexiglas box in the 37°C incubator. After the 2-hr incubation had been started, and 2-3 hr after the control incubation had been initiated, the control flasks were removed from the incubator.

Cell Removal from the Agar Wells (after exposure) - With the flask in its Plexiglas holder held in its normal flat incubation position, a needle catheter with stylus was inserted through the agar plug in the neck of the flask into the well towards the top of the well space; this served as an air vent. A second needle catheter was then inserted into the well at a slightly downward angle, so that it would reach to the far end of the well (near its bottom). After removing both styli, a 12-ml luerlock syringe was attached to this withdrawal catheter. After as much fluid as could be collected was withdrawn, the syringe was detached from the needle catheter, and the suspension was transferred into a 15-ml sterile conical plastic tube in a Plexiglas shielding box. A second syringe loaded with 5 ml of fresh medium was attached to the withdrawal catheter, and the medium was injected into the well. This wash suspension was then withdrawn into the same syringe. This was done to rinse out as many cells remaining in the well as possible. The syringe was detached from the needle catheter, and the cells were added to the initially withdrawn volume. After these 2 steps were completed for one flask, the styli were returned to the 2 needle catheters, and they were then removed and placed into sterile 15-ml tubes. After the first flask was discarded, these same 2 needle catheters were inserted into the second and third flasks of the set.

Washing of the Collected Cell Suspension - After the cell suspensions had been collected (separately) from the 3 flasks of the set, the tubes were centrifuged for 5 min at 800 rpm in a Beckman PR-J refrigerated centrifuge. The supernatant was removed by sterile pipet and transferred into a labeled tube (for subsequent radioactivity counting to determine leaching of the ^{32}P from the agar into the cell suspension), and the cells were resuspended in 5 ml of F12CM10 for washing. The cells were pelleted again by centrifugation, and this 5-ml rinse supernatant was transferred by pipet into separately labeled tubes. The cells were then resuspended in the tube with a final 3-ml volume of F12CM10 and placed in ice until all 3 of the sets were similarly collected.

Cell Counting and Plating for Colony Formation - After the final cell resuspension, the cell density in each of the final 3-ml volumes was determined by hemocytometer count. Because of residual agar in the cell pellets, electronic counting could not be performed. For each of the 9 separate treatment flasks, 8 T-25 flasks were subsequently inoculated with 200 cells (per flask) and incubated for (initial) survival determination using the standard protocol.

Results

The results of this experiment are summarized in Table 4. The first observation is that the cloning efficiency of the control cells is below that typically observed for this cell line, when a comparison is made to standard surface attached treatment studies (a cloning efficiency of 50%, compared to an expected 80%). Each of the values for cloning efficiency appearing in Table 4 is the mean of the 8 assay flasks seeded for each treatment flask.

TABLE 4. SURVIVING FRACTION OF AS52 CELLS EXPOSED TO ^{32}P-AGAR AT 50 μCi/ml AT 37°C

Treatment	Cells Plated/ Flask	Mean Colonies/ 8 Flasks	Mean CE (Control)	Mean Fractional Survival/ 8 Treatment Flasks	Mean Fractional Survival/ Treatment
Control A	199	105			
Control B	203	98	.499		
Control C	203	99			
2 hr Exposure A	199	85		.856	.910
2 hr Exposure B	204	98		.963	
2 hr Exposure C	199	128		1.289*	
4.6 hr Exposure A	198	67		.678	.750
4.6 hr Exposure B	200	82		.822	
4.6 hr Exposure C	198	103		1.042*	

* These values were not used in the calculation of the average because the Mean Survivals/8 Treatment flasks of these samples were greater than 1.0.

The recovered cell counts were very low in both samples excluded.

Each of the surviving fractions is (with one exception) the average of the means of the 3 independent treatment flasks for that exposure. It can be seen that after an exposure of 2 hr, the average surviving fraction was 0.91, while after the 4.6-hr exposure, the surviving fraction was .75. The latter value may actually be lower, since one of the values included in the calculation of the mean for the latter appears to be an outlier.

VIII. Objective: To determine AS52 cell survival and mutagenicity as a result of ^{32}P exposure in the agar well system, when the radioactivity concentration used was 75 μCi/ml, and one of the incubation times was increased to approximately 6 hr.

Hypothesis: Increasing the exposure time to a maximum of approximately 6 hr, with a 75 μCi/ml radioactivity concentration in the agar layer, would result in a decrease of cell survival to below the 75% previously observed, and result in measureable mutagenesis.

Methods

The agar flask preparation for this experiment was performed as previously described. Four radioactivity flasks were prepared several days before the experiment, so that the radioactivity concentration at the time of addition of the cell suspension to the flasks would be 75 μCi/ml (1.5 mCi/treatment flask). Four nonradioactivity flasks were also prepared. Two served as controls, and 2 were used for treatment of the cells with the known chemical mutagen EMS. The flasks were initially refrigerated, and on the evening before the exposure, were placed into the 37°C incubator for warming prior to cell addition.

The cell suspension (1×10^6 cells in 8 ml) was first added to the agar wells of the 2 control flasks; these were then incubated for 4.2 hr or 6.8 hr. The 2 exposure times were longer than originally planned and resulted from the complexity of the protocol (and safety considerations). After starting the control incubations, cell suspension was added to the 4 agar well ^{32}P flasks; 2 flasks were incubated for 3.5 hr and 2 for 6.8 hr. Finally, cells were added to the 2 EMS flasks, which were incubated for 3.6 hr or 6.8 hr. The addition of the cells to the agar wells in the flasks was performed using the syringe addition technique described above.

For the EMS treatment, an aliquot (0.216 ml) of a sterile 50 mg/ml EMS solution was added to 18 ml of the starting cell suspension. This yielded a final EMS concentration of 600 μg/ml. The EMS was added immediately prior to inoculation of the cell suspension into the agar wells.

At the end of the incubation intervals indicated, the cells were removed from the flasks using the cell removal procedure already described. The cells were washed, resuspended, and counted. A small volume of cells was diluted and plated for survival determination; the remaining cells were added to flasks already containing 5 ml of fresh warm medium for a 6-day expression incubation. Both survival and mutagenicity assays were performed as described earlier. This immediate seeding of cells for survival determination is at variance from the standard mutation assay, which alows for an overnight incubation prior to assay.

Results

These are presented in Table 5. The cloning efficiency was 0.69 in the 4.2-hr control flask, and 0.42 in the 6.8-hr control flask. These lower values for C.E., compared to the standard plate assay, were again cause for concern. The surviving fraction values for the 3.5-hr ^{32}P exposed cells and the 3.6-hr EMS treated cells were calculated using the 4.2-hr control flask cloning efficiency as the 100% survival value. The surviving fraction values for the longer exposure times were calculated using the cloning efficiency of the 6.8-hr control incubation as the 100% survival value. For the 3.6-hr and 6.8-hr EMS incubations, the percent survivals were 83% and 66%. For the 3.5-hr ^{32}P exposure, the average percent survival of the 2 flasks was 35.3%, while after the 6.8-hr ^{32}P exposure, the average percent survival was reduced to 7.2%. These values show increased toxicity for the 75 μCi/ml ^{32}P agar, when compared to the 91% survival at 2 hr and the 75% survival at 4.6 hr for the 50 μCi/ml radioactivity concentration of the earlier experiment. The goal (and hypothesis) of increased toxicity was achieved. In Table 5, the mutagenicity results for the experiment are also presented. For the EMS, at a final treatment concentration of 600 μg/ml, the frequencies were 89 x 10^{-6} and 234 x 10^{-6} induced mutants/clonable cell. The ^{32}P β^- exposure clearly resulted in mutation of the cells, with a value of 104 x 10^{-6} induced mutants/clonable cell for a 3.5-hr exposure and an average value (2 flasks) of 787 x 10^{-6} induced mutants per clonable cell for the 6.8-hr exposure.

IX. Objective: To determine the heat sensitivity of CHO and AS52 cells.

Hypothesis: The heat sensitivity, as measured by cell survival, after plastic surface attachment and heating as a monolayer, will be the same for the wild-type CHO and the transformed AS52 cells, similar to our observations for γ-ray sensitivity.

Methods

Survival Determination

For both cell lines, monolayer cultures of cells were resuspended on the day of the experiment using 0.25% trypsin, and the cell density determined. Cells were seeded into T-25 tissue culture flasks; the final volume was adjusted to 5 ml to insure constant heating kinetics. The number of test cells plated into each flask was varied, in order to obtain approximately 100 colonies after each treatment. The cell density was maintained at 10^5 cells/flask by adding lethally irradiated (25 Gy) feeder cells to the flasks (to be used) 24 hr prior to plating the test cells. For each treatment, 8-10 replicate flasks were plated. After the initial test cell distribution, the flasks were returned to a 37°C incubator for 120 min; this allowed the test cells to attach to the plastic before heating. After heating (as described below), the flasks were incubated at 37°C for 7 to 14 days to allow formation of macroscopic colonies of more than 50 cells. Colonies were fixed and stained with crystal violet (prepared in 95% ethanol) and counted for survival determination. Surviving fraction was corrected for plating efficiency (80-90%) and cellular multiplicity. To determine multiplicity, 5 x 10^3 cells were seeded into T-25 flasks and allowed to attach (during the same 2-hr attachment time as the test cells). At the time of heating, replicate multiplicity assay flasks were fixed with 3:1 methanol:glacial acetic acid.

TABLE 5. SUMMARY OF INITIAL SURVIVING FRACTION (DAY 0), FRACTION OF CLONABLE CELLS (DAY 6), AND MUTAGENICITY, FOR ^{32}P β^- EXPOSURE OF AS52 CELLS FOR 3.5 OR 6.8 HR (37°C), 75 μCi/ml = 1.5 mCi/FLASK

Exposure Condition (Replicate A or B)	Initial (Day 0) Cloning Efficiency (Mean ± SE)	Initial (Day 0) Surviving Fraction (Mean ± SE)	Day 7 Cloning Effic. (Mean ± SE)	Day 7 Fraction of Clonable Cell (Mean ± SE)	Mut. Freq/ Clonable Cell ($x10^{-6}$)	Induced Mut. Freq. ($x10^{-6}$)
Control: 4.2 hr	.693±.027	1.0	.841±.017		30.94	-
^{32}P: 3.5 hr-A	.242±.010	.349±.014	.853±.038	1.014±.044	134.66	103.72
^{32}P: 3.5 hr-B	.247±.010	.357±.014	-	-	-	-
EMS: 3.6 hr	.575±.009	.830±.013	.839±.013	.992±.011	120.32	89.38
Control: 6.8 hr	.424±.026	1.0	.951±.017	-	33.66	-
^{32}P: 6.8 hr-A	.035±.007	.083±.016	.723±.065	.760±.068	459.07	425.41
^{32}P: 6.8 hr-B	.017±.008	.050±.016	.524±.019	.551±.020	1182.81	1149.15
EMS: 6.8 hr 600 μg/ml	.276±.020	.655±.048	.811±.026	.853±.027	267.31	233.95

SE = Standard Error

At a later time, the cells were stained with 5% Giemsa for 5 min; cellular multiplicity was calculated as the average number of cells per colony forming unit.

Hyperthermia Treatment

Thirty minutes prior to treatment, the necks of the flasks were sealed with paraffin wax to prevent leakage. Hyperthermic treatments were conducted by total immersion of the flasks into Plexiglas water baths regulated to predetermined temperatures by Hetotherm controller/circulators ($\pm$ 0.03°C). The flasks were placed into a 37°C water bath for 5 min immediately before and again after heating, to insure constant heat-up and cool-down kinetics. After this heat treatment, the flasks were returned to a 37°C incubator for colony formation.

Results

The survival of CHO cells, heated at 42-45.5°C for varying times, is shown in Figure 9. At 42 and 42.5°C, thermotolerance develops after 4 to 6 hr of heating. At temperatures above 43°C, there is a decreased shoulder (D_q) and increased slope (decreased D_o) with increasing temperature (Fig. 10). Survival curves were also obtained for AS52 cells heated for varying times from 41 to 42.5°C (Fig. 11). In contrast to the data obtained with the CHO cells, the AS52 cells did not appear to develop thermal tolerance during heat treatments of up to 10 hr at 42.5°C, although the curve is biphasic. When survival after heating at 42 or 42.5°C is compared for the 2 cell lines (Fig. 12), the AS52 cells appear to be more heat sensitive. Further studies are necessary to compare AS52 sensitivity with the heat sensitivity of its parent line, for comparison to that described here for a different line of CHO cells. It also is possible that these differences may be partially attributed to the different media used for the 2 cell lines.

X. Objective: To determine the viability of AS52 cells after heat treatment in agar wells. Based on the earlier observations of decreased cloning efficiency at 37°C (control), this experiment was designed to determine our ability to use this system for simultaneous RFR/heating and ionizing radiation exposures.

Hypothesis: That the survival of AS52 cells would be decreased with increasing temperature, when the cells were incubated (without ionizing radiation exposure) for 6 hr in agar wells.

Methods

In performing these studies, 12 agar well flasks were prepared as previously described (using F12 medium without serum). A stock flask of AS52 cells, previously prepared, was trypsinized to detach the cells. Since 6 test flasks, each seeded with 1 x 10^6 cells would be used the next day, 2 x 10^6 cells of this cell suspension were seeded in 15 ml in a spinner flask. This was done so that the membranes of the cells would recover from any trypsin damage prior to the heat treatment on the following day. The necessary 4-in catheters and small test tubes were autoclaved, the agar flasks were placed for overnight incubation in the 37°C incubator, and the water baths for immersion heating were set to the correct temperature.

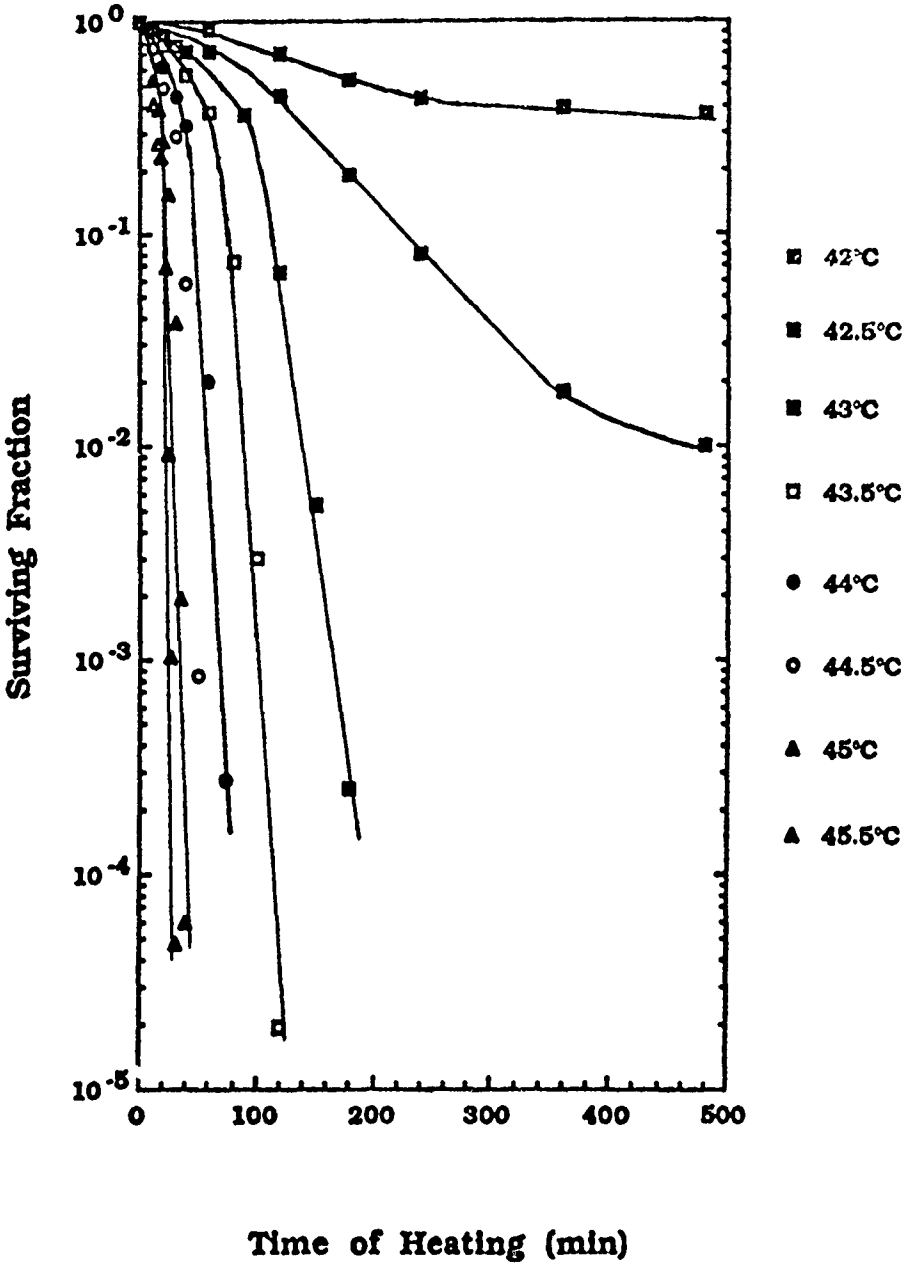

Figure 9. Survival of CHO cells, heated at 42.0-45.5°C for varying times. Cells were preplated at varying cell densities, heated and then incubated at 37°C for 7 to 14 days to allow for colony formation.

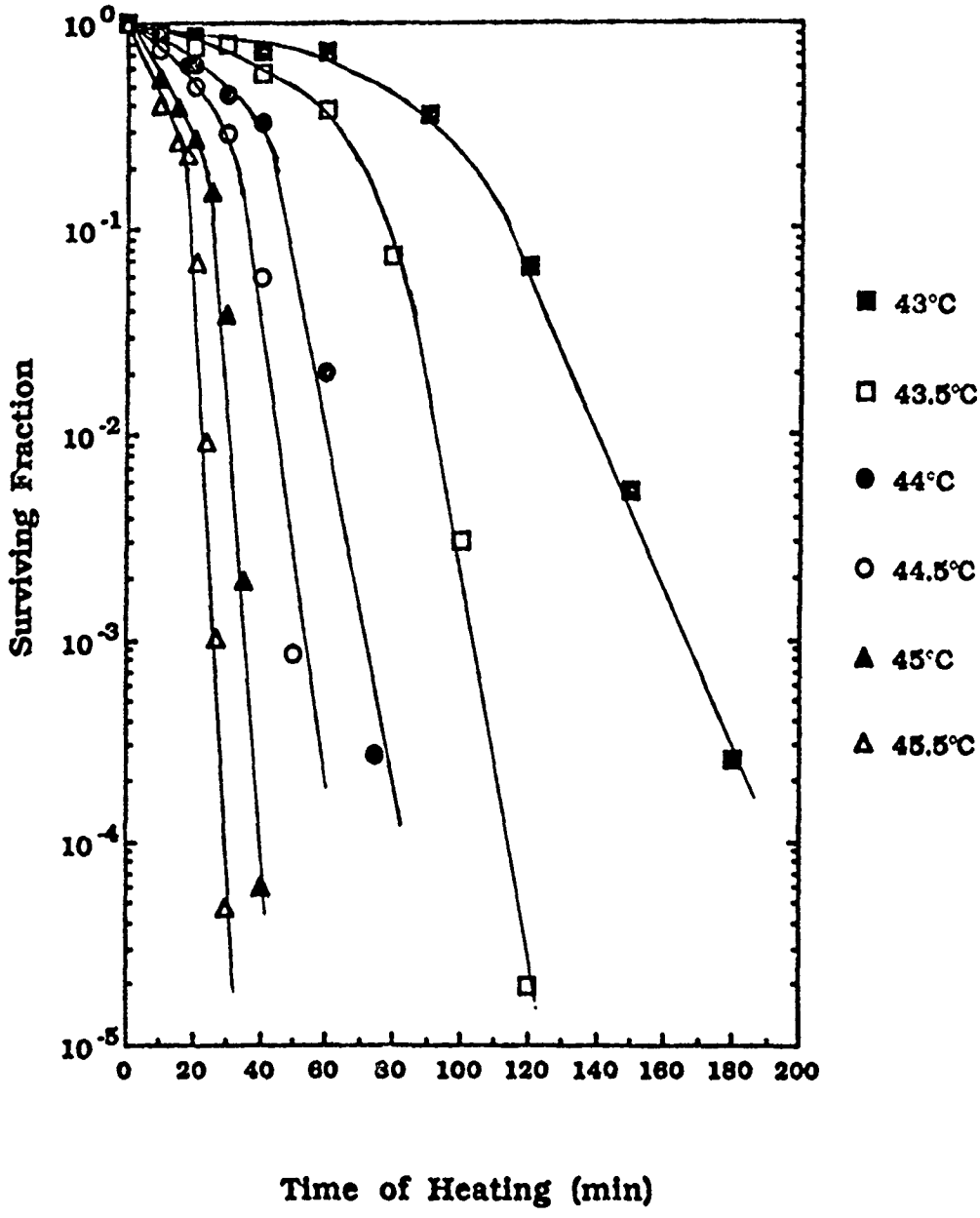

Figure 10. Survival of CHO cells heated at 43.0-45.5°C for varying times.

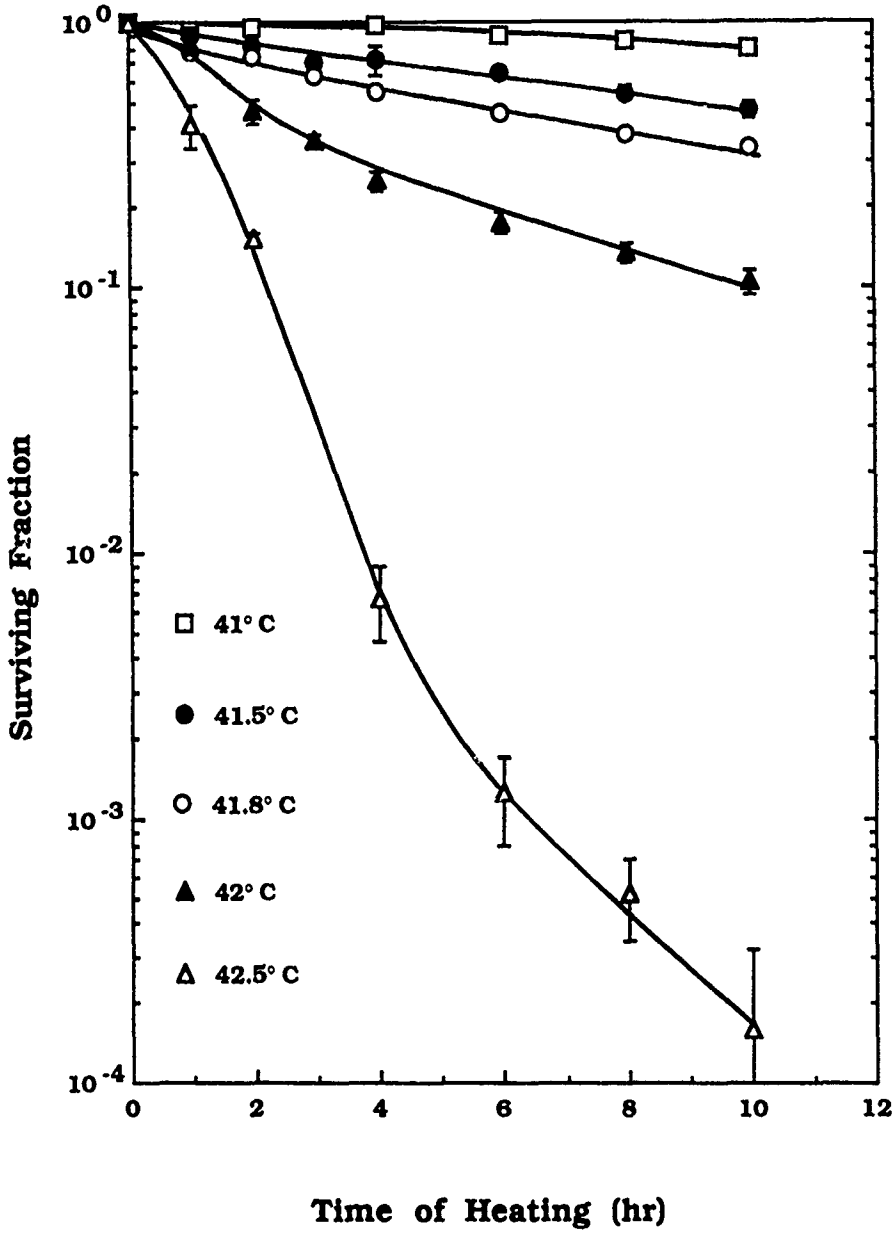

Figure 11. Survival of AS52 cells heated at 41.0-42.5°C for varying times.

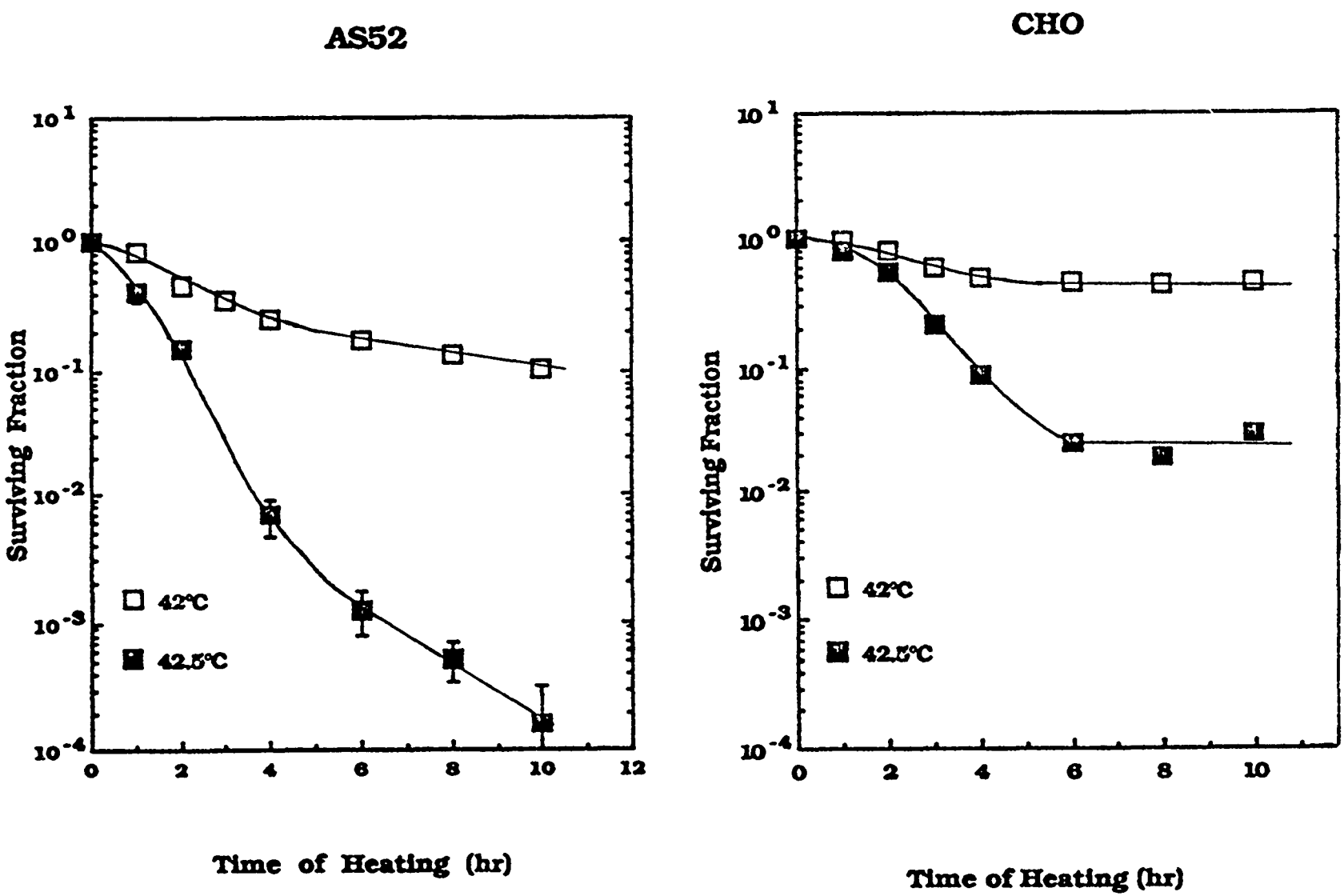

Figure 12. Comparison of survival of CHO and AS52 cells after heating at 42 and 42.5°C.

On the next day, the cells in suspension in the spinner flask were counted, and diluted by adding 6.2×10^6 cells to 49.6 ml of F12 medium with 10% FBS. This yielded a final density with 1×10^6 cells in each 8 ml volume. The cells were then added into the agar flasks, the cap and neck was sealed with paraffin, and the flasks placed in racks and immersed in 37°C water bath for at least 10 min. The flasks were subsequently immersed in the pre-set water baths for the desired time, and incubated for 6 hr. The remainder of the procedure is as previously described; the cells were seeded on lethally irradiated feeder layers for survival determination. The exposures performed on this first experimental day included 37°C (2 flasks), 41°C (2 flasks), and 41.5°C (2 flasks). The procedure was repeated at higher temperatures 2 days later, and incubations were performed for 6 hr at 37°C (2 flasks), 42°C (2 flasks), and 42.5°C (2 flasks).

Results

A summary of the effects of agar well incubation of AS52 cells for 6 hr at different (and increasing) temperatures is presented in Table 6.

Discussion

Based on the results of the experiment, it is clear that the survival of the AS52 cells decreases with incubation in agar for 6 hr at increasing temperatures. For the performance of any 6-hr RFR exposure experiment where the temperature will increase, it would be necessary, based on these observations, to keep the maximum temperature at or below 41.5°C, to avoid agar-heat complication of the data. Furthermore, if one compares the survival of AS52 cells heated in monolayer culture (Fig. 11, Objective IX) with that of the same AS52 cell survival after heating in agar (Table 6), the cells heated in agar appear to be much more heat sensitive. For example, 6 hr at 41.5°C reduces fractional survival to 0.23 if cells are heated in agar, but for monolayer cells, the fractional survival of similarly heated cells is only reduced to 0.6. It is possible that the heat may have a direct effect on the agar, leading to a combined cytotoxic effect.

XI. Objective: To adapt the propidium iodide staining technique, previously described in the literature, for FACS 420 analysis of the cell types employed in our laboratory.

Background

The distribution of the DNA content of a population of *in vitro* cultured mammalian cells can be determined using the DNA intercalating dye propidium iodine. The amount of stain present in the cell is proportional to the amount of DNA present, which reflects whether a given cell is in the G1, S or G2/M stage of the cell cycle. The flow cytometric analysis of stained cells generates histograms which demonstrate the number(s) of cells with different DNA contents.

TABLE 6. SUMMARY OF HEAT TREATMENT OF AS52 CELLS FOR 6 HOURS IN AGAR WELLS*

Treatment (°C)	# cells seeded per dish (n=8)	Mean # of Colonies	Cloning Efficiency	Surviving Fraction Indiv. / Mean
37 A	125	105	.84 (.832)	1.0
37 B	105	87	.829	
41 A	200	53		.319 .351
41 B	135	43		.383
41.5 A	180	35		.234 .233
41.5 B	155	30		.233
	* * * * *			
37 A	145	96	.66 (.66)	1.0
37 B	159	105	.66	
42 A	147	3		.031
42 B	130	2.6		.030
42.5 A	125	0		
42.5 B	100	0		

* The upper and lower sections of the table are for experiments performed on sequential days. A and B are replicate independent incubation flasks.

Method

Cells to be analyzed were harvested and fixed in ice cold 70% ethanol. They were then stored at 4°C until the final preparation for flow cytometry. After such refrigeration, the cells were pelleted at 1000 rpm for 5 min in a Beckman PR-J Tabletop Centrifuge at 23°C. They were then resuspended by vortexing in 1 ml of Krishan's buffer. The latter consists of a solution of 0.1% sodium citrate containing 50 μg/ml propidium iodide, 0.3% NP-40, and 20 μg/ml RNAse. The cells were incubated in the buffer (in the dark) at 4°C for 60 min. They were then filtered through a 50-μm mesh screen before use.

For flow analysis, the cells were run through a 70-μm diameter ceramic orifice at a rate of 30 to 90 cells per second. Excitation of the propidium iodine stain was accomplished with a Spectra Physics Argon/Krypton laser operated at 300 mW, at a wavelength of 488 nm. Information from 10,000 cells were typically acquired per sample, and analyzed on a Becton-Dickinson Consort 30 Computer System.

Results

Representative plots of the untreated DNA distributions for CHO cells (upper panel) and human lymphoblastoid 244B cells (lower panel), two of the cell lines used in this project, are shown in Figure 13.

XII. Objective: To use the FACS 420 flow cytometry system for analysis of effects of selected heat treatments (temperature and time) on cell cycle distribution of mammalian cells.

Hypothesis: Heat treatment of 244B human lymphoblastoid cells will result in an alteration, measurable after post-heat treatment incubation at 37°C, of their distribution in the different stages of the cell cycle.

Methods

One preliminary experiment was conducted. Cells were seeded into T-25 flasks, at a cell density of 2.5×10^5 cells/ml in 5 ml of medium. The cells were incubated for varying time periods at different temperatures, ranging from 37 to 43.5°C. Cells were then incubated for 0 to 72 hr at 37°C after this heat treatment, before they were fixed and stained as described previously. One flask provided cells for each condition examined.

Results

The results of this study proved to be inconclusive. First, it was found that the fixation procedure employed did not prevent degradation of the samples, while refrigerated. Secondly, it was evident that damage to the cells due to the heat treatments also contributed to the difficulty in analysis.

Problem Resolution

The fixation problem has been addressed by switching to a citrate buffered ethanol fixative; this demonstrated superior performance in recent tests in our laboratory.

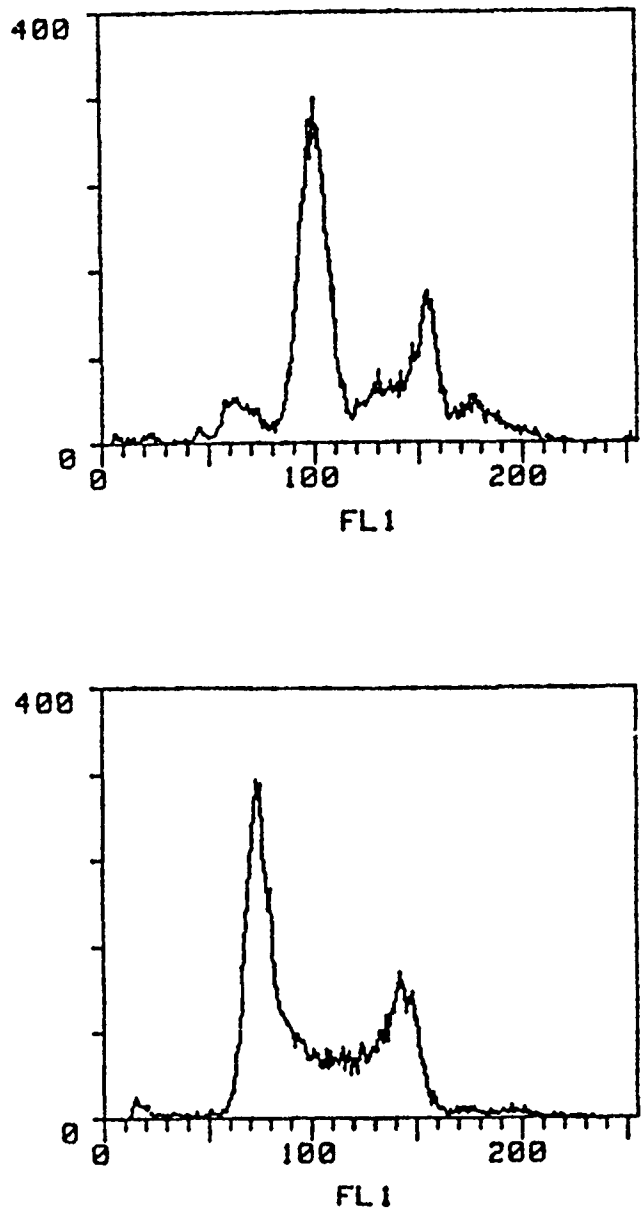

Figure 13. FACS 420 analysis after propidium iodide staining of CHO cells (upper panel) and human lymphoblastoid 244B cells (lower panel).

The second part of the problem has been addressed by the acquisition of a computer program which will allow analysis of the data by comparison of the relative percentages of experimentally treated data with that of controls. The program initially used, the S-Fit program used in the Consort 30 system, relied on a specific mathematical equation with preset limits of data acceptability.

Another change which could play a role in successful analysis is an increase in the recovery period post-heat treatment and prior to analysis. While this change might help analysis, it would not be helpful in analysis of shorter term heat treatment effects.

B. COMPLETED STUDIES

We have completed a series of studies of the ability of RFR in the microwave range: 1) to cause genotoxic damage or interfere with the ability of mammalian cells to repair DNA damage caused by ultraviolet light exposure; and 2) to alter the extent of genotoxic damage resulting from a simultaneous exposure to known genotoxic chemicals (selected because of their ability to interact with cellular DNA via different mechanisms of action).

1. Genotoxic Activity of RFR Alone

A series of investigations has been undertaken to examine the ability of RFR at different frequencies, including 350, 850, 1200, and 2450 MHz, at low and moderate power levels (and specific absorption rates - SARs), delivered either as continuous or pulsed wave exposures, to cause genotoxic events in mammalian cells. The cell lines were selected based on their appropriateness for the genotoxic endpoint examined. These have included induction of DNA repair synthesis in normal human diploid fibroblasts, mutation at the thymidine kinase locus in mouse leukemia L5178Y cells, and sister chromatid exchange (SCE) and chromosome aberration induction in Chinese hamster ovary (CHO) cells. At the lower power densities, the temperature of the incubation medium was constant at 37°C or 39°C during the several hr exposure period. In the studies performed at moderate exposure values, temperatures up to 40°C were achieved.

Methods

RFR Exposure and Temperature

The exposure and temperature monitoring methods have previously been described for the DNA repair studies (Meltz et al., 1987), and the mutation (Meltz et al., 1989) and SCE chromosome aberration induction studies (Ciaravino et al., 1987; Kerbacher et al., 1990). The RFR exposure parameters for these experiments are described as follows:

RFR exposures at 350 MHz for the DNA repair studies were performed in a Transverse Electric Mode (TEM) Transmission Cell (NARDA).

RFR exposures at 850, 1200, and 2450 MHz were performed in a 40 x 20 x 10 ft anechoic chamber (No.2) in the Radiofrequency Research Laboratory at the U.S. Air Force School of Aerospace Medicine. The air temperature in the

chamber was initially maintained at 37°C. The continuous wave (CW) or pulsed wave (PW) RFR was transmitted from a rectangular antenna horn in a vertically downward direction.

The power densities, specific absorption rates (SARs), mode of exposure, frequency, and duty factor (for PW exposures) varied among the types of experiments; the values are reported for each experimental series.

The horn to water bath distance was 1.6 m, thus placing the flasks or dishes containing the cells to be exposed in the near field, just short of the far field. For the different types of genotoxic assays, different water bath exposure systems were designed and used. For the DNA repair studies, cells were exposed in 24 x 24 cm plastic tissue culture dishes floating in a stationary rectangular water bath; in the SCE, chromosome aberration and mutation studies, cells were exposed in 25 cm^2 tissue culture flasks (T-25 flasks) immersed in a large square water bath, but positioned as inserts in the bottom side of a continuously rotating Styrofoam float. The latter was designed to insure the homogeneity of the exposure (Meltz et al., 1988).

Temperature Monitoring

The temperature was continuously monitored during the RFR exposure period using non-RFR-interactive Vitek probes and a BSD-200 Thermometry system. In the DNA repair studies, the medium temperature during all exposures at all frequencies remained constant at 37°C ± 0.5°C. In the studies performed at higher power densities at 2450 MHz, the medium temperature always increased. A representative temperature plot over a 2 hr period is shown in Figure 14. A rapid rise in medium temperature over the first 20 min was followed by a slow approach to RFR-induced maximum temperatures at 2 to 4 hr approaching 40°C.

RFR Exposure Parameters

At 350 MHz, for the DNA repair studies, the exposure parameters were:

RFR Source: MCL Model 15022 RF Power Generator			
Pulse Repetition Rate:		5000 pps	5000 pps
Pulse Width:		10 μs	10 μs
Duty Factor:		0.05	0.05
Power Density:	-Avg.:	1 mW/cm^2	10 mW/cm^2
	-Peak:	20 mw/cm^2	200 mW/cm^2
SAR:			0.39±0.15 W/kg

AT 850 MHz, for the DNA repair studies, the exposure parameters were:

RFR Source: COBER Electronics Inc. Model 15022 High Power Microwave Generator and MCL Model 10110 Amplifier			
Pulse Repetition Rate:		5000 pps	5000 pps
Pulse Width:		10 μs	100 μs
Duty Factor:		0.05	0.5
Power Density:	-Avg.:	1 mW/cm^2	10 mW/cm^2
	-Peak:	20 mw/cm^2	20 mW/cm^2
SAR:			4.5±3.0 W/kg

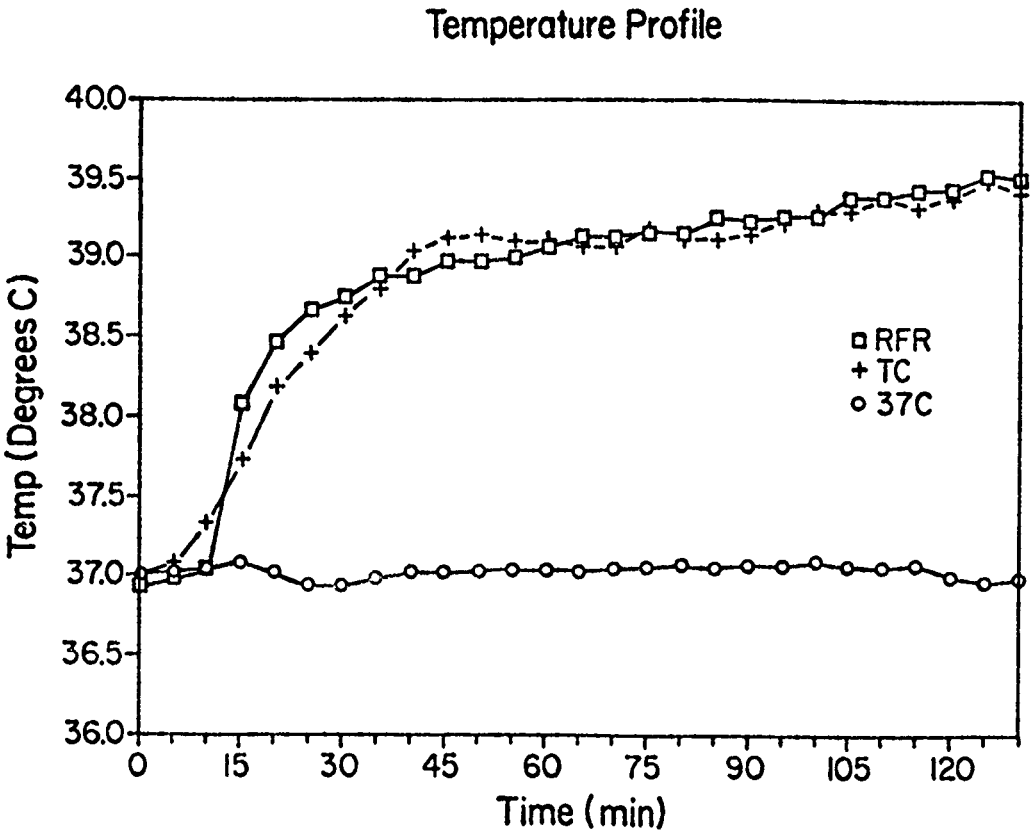

Figure 14. Temperature profile in flask during 2-hr RFR exposure period.

At 1200 MHz, for the DNA repair studies, the exposure parameters were:

RFR Source: COBER Electronics Inc. Model 1831 High Power Generator		
Pulse Repetition Rate:	80,000 pps	80,000 pps
Pulse Width:	3 μs	3 μs
Duty Factor:	0.24	0.24
Power Density:-Avg.:	1 mW/cm^2	10 mW/cm^2
-Peak:	4.2 mW/cm^2	41.7 mW/cm^2
SAR:		2.7$\pm$1.6 W/kg

At 850 MHz, for the SCE and Chromosome Aberration studies, the exposure parameters were:

Avg. Net Forward Power:	200 W
Pulse Repetition Rate:	40,000 pps
Pulse Width:	6.5 μs
Duty Factor:	0.26
Power Density:-Avg.	18 mW/cm^2
SAR:	14.4 W/kg

At 1200 MHz, for the SCE and Chromosome Aberration studies, the exposure parameters were:

Expt. No.:	1	2	3
Avg. Net Forward Power:	300 W	220 W	240 W
Pulse Repet. Rate:	40,000 pps	40,000 pps	40,000 pps
Pulse Width:	6.5 μs	6.5 μs	6.5 μs
Duty Factor:	0.26	0.26	0.26
SAR:			24.33 W/kg

AT 2450 MHz, for the mutagenesis, SCE, and Chromosome Aberration studies, the exposure parameters were:

RFR Source: COBER Electronics Inc. Model 1831 High Power Microwave Generator and Hewlett-Packard Model 8011A Pulse Generator		
Pulse Repetition Rate:	25,000 pps	25,000 pps
Pulse Width:	10 μs	10 μs
Duty Factor:	0.25	0.25

For L5178Y Mutation Experiments:	**Series 1**	**Series 2**	
Forward Power (Watts):	600	500 or 600	
Power Density:-Avg. (mW/cm^2):	48.8	87	65
Spec. Absorp. Rate (SAR:W/kg):	30	40.8	40

For Sister Chromatid Exchange Experiments:

Forward Power (Watts):	600
Power Density:-Avg. (mW/cm^2):	49
Spec. Absorp. Rate (SAR: W/kg):	33.8

For Chromosome Aberration Experiments:

Forward Power (Watts):	600
Power Density:-Avg. (mW/cm^2):	49
Spec. Absorp.	33.8

Cell Line and Genetic Assay Protocols

The description of the MRC-5 human diploid fibroblast cell line, and the protocol for the DNA repair replication assay protocol, has been reported (Meltz et al., 1987). The protocols for the use of L5178Y cells in the mammalian cell mutagenesis assay have previously been described (Meltz et al., 1989), as has the protocol for measurement of SCE induction in CHO cells (Ciaravino et al., 1987) and chromosome aberration induction in CHO cells (Kerbacher et al., 1990).

For the chromosome aberration studies at the frequencies of 850 and 1200 MHz, the same procedure for RFR exposure as described in Kerbacher et al., 1990 was used, but an alternative to the in situ coverslip technique described therein was employed. After exposure at these 2 frequencies, the cell monolayer was washed, the cells were detached, and they were then fixed in suspension. They were subsequently dropped for cytogenetic analysis using standard techniques.

Results of DNA Repair Studies

Figures 2, 3, and 4 of Meltz et al., 1987 show the lack of an effect of RFR at the frequencies of 350, 850, and 1200 MHz on UV induced DNA repair, whether the comparison is made at 1, 2, or 3 hr of the DNA repair period, a simultaneous exposure (at 37°C) to either CW or PW radiation at these frequencies and power densities of 1, or 10 mW/cm^2 had no effect on the extent of repair radioactivity incorporated. As also described (Table 2; Meltz et al., 1987), a similar absence of an effect was reported if the cells were incubated at 39°C during the repair labeling period.

In Meltz et al. (1987), the ability of RFR to directly cause DNA damage, leading to induction of DNA repair, was also examined. Table 7 clearly demonstrates the inability of RFR, for a 3-hr exposure and repair labeling interval, to induce DNA repair at any of the 3 frequencies as studied, even if the cells are incubated with a "thermal stress" temperature of 39°C during the RFR exposure.

TABLE 7. ABSENCE OF RFR INDUCTION OF DNA REPAIR IN HUMAN MRC-5 CELLS

Frequency	Mode	Temperature	Exposure dpm/μg	Control DNA
350 MHz	CW	37°C	72 (10)*	76
850 MHz	CW	37°C	299 (10)	284
1200 MHz	CW	37°C	444 (10)	421
350 MHz	PW	37°C	287 (10)	259
850 MHz	PW	37°C	224 (10)	120
1200 MHz	PW	37°C	867 (10)	1259
1200 MHz	PW	37°C	398 (10)	420
350 MHz	PW	39°C	765 (5)	835
850 MHz	PW	39°C	714 (10)	824
850 MHz	PW	39°C	476 (10)	327

*The value in parentheses is the power density in mW/cm^2

TABLE 8. ABSENCE OF INDUCTION OF SCEs BY PULSED WAVE RFR AT THREE FREQUENCIES

Exposure to 2450 MHz PW

NUMBER OF SCEs PER CELL ± S.E.M.

Expt. No.:	1	2	3	4
EXPOSURE				
37°C	10.10±0.48	8.10±0.38	9.94±0.48	17.22±0.72
37°C + MMC	18.94±0.60	21.84±0.85	29.48±1.09	25.42±1.01
TC	10.54±0.42	8.90±0.45	10.84±0.45	16.94±0.77
RFR	10.90±0.46	9.88±0.39	9.56±0.39	13.78±0.56
37°C	7.92±0.36	10.12±0.47	9.48±0.40	8.22±0.37
37°C + ADM	16.90±0.74	17.38±0.83	13.42±0.80	13.86±0.54
TC	8.72±0.38	8.10±0.40	8.38±0.45	8.94±0.43
RFR	8.70±0.39	8.90±0.48	9.06±0.43	8.10±0.44

(Cells were treated with Adriamycin (ADM) at 7.75×10^{-7} M in place of MMC)

Exposure to 850 MHz PW

NUMBER OF SCEs PER CELL + S.E.M.

Expt. No.:	1	2	3
EXPOSURE			
37°C	8.23±0.41	7.33±0.57	7.10±0.39
37°C + ADM	13.00±0.81	13.73±0.86	8.63±0.50
TC	7.17±0.44	8.43±0.54	6.50±0.47
RFR	7.30±0.49	7.83±0.39	6.90±0.43

(Cells were treated with ADM at a very low concentration, 1×10^{-8} M)

Exposure to 1200 MHz PW

NUMBER OF SCEs PER CELL + S.E.M.

Expt. No.:	1	2	3
EXPOSURE			
37°C	12.73±0.69	12.17±0.73	8.20±0.59
37°C + ADM			14.60±1.15
TC	13.33±0.79	9.83±0.51	8.37±0.52
RFR	11.53±0.63	9.57±0.54	11.27±0.54

(Cells were treated with 1×10^{-8} M ADM)

Examination of RFR Induction of Chromosome Aberrations In CHO Cells

In this type of study, the 37°C control provides a baseline value for the "spontaneous" occurrence of chromosome aberrations in CHO cells. The chemical mutagen serves as a positive control. The TC serves to indicate whether any effect observed for RFR is due to the RFR, or simply due to elevating the cells to a higher temperature (using a water bath).

As can be seen in the information summarized in Table 9, for pulsed wave exposures at 800 MHz and 1200 MHz, and Table 10 for 2450 MHz, no evidence exists that RFR induces chromosome aberrations. There were no statistically significant differences between the number of chromosome aberrations in the RFR exposed cells and the 37°C or TC cells.

Examination of 2450 MHz RFR Induction of Mutations in L5178Y Cells

In this type of study, the 37°C control provides a baseline value for the "spontaneous" occurrence of mutations at the thymidine kinase locus in the heterozygote L5178Y cells. The chemical mutagen treatment serves as a positive control. The TC serves to indicate whether any effect observed for RFR is due to the RFR, or simply due to elevating the cells to a higher temperature (using a water bath).

As can be seen in the information summarized in Table 11 below, for pulsed wave exposures at 2450 MHz, no evidence exists that RFR induces mutations in L5178Y cells.

CONCLUSIONS

As is evident from the experimental information summarized, there is no statistically significant evidence, or in fact even suggestive evidence, that RFR in the microwave range, of SARs and power densities at or above the recommended exposure guidelines, where medium temperatures (continuously monitored) range from 37°C to 40°C, can cause genetic damage of an inheritable nature.

2. Alteration of genotoxic activity of chemicals by simultaneous RFR exposure to pulsed wave RFR at 2450 MHz.

A second hypothesis that we have examined in a series of completed experiments is that RFR exposure during a chemical treatment of mammalian cells can result in an alteration of the extent of genotoxic action caused by the chemicals. The possibility that this could occur has been examined for: a) mammalian cell mutation, b) SCE induction, and c) chromosome aberration induction.

Results

Mammalian Cell Mutation

Mitomycin C is known to be a bi-functional alkylating agent which can cause DNA cross-links. When cells were exposed simultaneously to RFR and MMC (Meltz et al., 1989; SAR = W/kg, Power Density = 48.8 mW/cm^2) no effect of

TABLE 9. ABSENCE OF INDUCTION OF CHROMOSOME ABERRATIONS BY 850 MHz AND 1200 MHz PW RFR

850 MHz PW

Experiment 1	ABERRATION TYPE (PER 100 CELLS)						
Experimental Condition	BRK	RAD	ID	DIC	RF	AC	N
37°C Control	4	0	0	0	0	28	500
RFR Exposed	0	0	0	2	0	16	300
Temperature Control	0	0	0	4	0	34	400
Adriamycin Treated	2	0	0	0	0	0	214

Experiment 2	ABERRATION TYPE (PER 100 CELLS)						
Experimental Condition	BRK	RAD	ID	DIC	RF	AC	N
37°C Control	0	0	0	0	0	18	400
RFR Exposed	8	0	0	0	0	10	400
Temperature Control	4	0	0	2	0	8	400
Adriamycin Treated	14	0	0	0	0	32	400

TABLE 9 (Cont'd.):

1.2 GHz

ABERRATION TYPE (PER 100 CELLS)

Experimental Condition	BRK	RAD	ID	DIC	RF	AC	N
37°C Control	4	0	0	0	0	8	500
RFR Exposed	0	0	0	0	0	0	400
Temperature Control	0	0	0	0	0	4	400
Adriamycin Treated	2	0	0	0	0	6	300

Summary of abbreviations: BRK = chromosome break; RAD = radicals; ID = deletion; DIC = dicentric; RF = ring plus fragment; AC = acentric fragments and double minutes; and N = number of cells scored

TABLE 10. ABSENCE OF INDUCTION OF CHROMOSOME ABERRATIONS BY 2450 MHz PW RFR

Experimental Condition	Aberration type (per 100 cells)[a]				
	tg	tb	sg	sb	f
37°C	1.5 ± 0.3	0.7 ± 0.2	3.5 ± 0.5	1.1 ± 0.3	1.1 ± 0.2
rf Radiation	1.9 ± 0.2	0.8 ± 0.2	3.1 ± 0.4	1.1 ± 0.3	1.1 ± 0.3
Temperature Control	1.9 ± 0.3	1.0 ± 0.2	3.5 ± 0.4	1.5 ± 0.4	1.0 ± 0.2
MMC (0.1 μg/ml)	20.7 ± 1.2	20.1 ± 3.5	34.6 ± 2.2	5.7 ± 1.1	8.8 ± 0.8

Experimental Condition	Aberration type (per 100 cells)				
	cr	min	td	r	d
37°C	0	1.9 ± 0.4	0	0.1 ± 0.1	0
rf Radiation	0	0.8 ± 0.3	0	0.1 ± 0.1	0
Temperature Control	0	1.0 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0
MMC (0.1 μg/ml)	1.4 ± 0.4	7.6 ± 0.4	0.3 ± 0.2	0.8 ± 0.4	0.2 ± 0.1

Aberration summary

Experimental Condition	Percentage Aberrant cells	Total Aberration[b] types (per 100 cells)	Total Aberration events (per 100 cells)	Aberration Events per aberrant cell
37°C	9.4 ± 0.5	10.0 ± 0.6	10.1 ± 0.6	1.07 ± 0.02
rf Radiation	8.0 ± 0.6	8.9 ± 0.8	9.0 ± 0.8	1.10 ± 0.02
Temperature Control	9.6 ± 0.6	10.0 ± 0.6	10.1 ± 0.6	1.05 ± 0.02
MMC (0.1 μg/ml)	62.8 ± 1.5	100.1 ± 3.0	102.6 ± 3.0	1.66 ± 0.06

The above data represent pooled results from seven experiments comprising 2100 cells in each condition (100 mitotic figures from each of three replicate slides per experiment), and are expressed as mean ± SEM. [a]Abbreviations are: tg-chromatid gap, tb-chromatid break, sg-chromosome gap, sb-chromosome break, f-fragment, cr-complex rearrangement, min-minute, td-terminal chromatid deletion, r-ring, and d-dicentric. [b]Total aberration types per 100 cells was summed using all of the raw data prior to establishing the mean. [c]MMC values represent pooled results from three experiments comprising 900 cells in each condition.

TABLE 11. ABSENCE OF MUTATION INDUCTION IN L5178Y CELLS:

EXPOSURE TO 2450 MHz PW RFR

Series 1

NUMBER OF MUTANTS PER 10^6 VIABLE CELLS ± S.D.

Expt. No.:	1	2	3	4	5
EXPOSURE					
37°C	59.5±0.85	37.7±3.24	98.4±14.2	100.3±1.56	91.0±14.4
TC	54.6±3.82	37.9±16.0	75.8±8.63	89.6	111.7
RFR	48.6±4.45	56.1±38.6	102.3±8.06	99.6±13.5	100.7±15.7

In the above experiments, the positive control mutagen, EMS, gave induced mutant frequencies (EMS mutants per 10^6 viable cells - 37°C) of 1,423; 3,527; 1,937; 1,626; and 1,848, in experiments 1 - 5, respectively.

Series 2

NUMBER OF MUTANTS PER 10^6 VIABLE CELL ± S.D.

Expt. No.:	1	2	3
EXPOSURE			
37°C	72.6±9.8	104.2±29.1	77.9±16.1
RFR	69.2±6.0	104.0±30.1	84.0±8.6

RFR on MMC induced mutation of L5178Y mouse leukemic cells of the thymidine kinase locus was observed.

The second chemical studied was proflavin, a drug known to intercalate with DNA. Again, when cells were exposed simultaneously to RFR (SAR = 40.8 ± 13.4 (SD) W/kg or 40 W/kg at power densities of 87 or 65 mW/cm^2), no effect of the RFR on the proflavin induced mutagenicity was observed (Meltz et al., 1990).

SCE Induction

Previously reported research from our laboratory (Ciaravino et al., 1987) has demonstrated, during treatment of CHO cells for 2 hr with mitomycin C, that simultaneous exposure to 2450 MHz pulsed wave RFR (SAR = 33.8 W/kg/ Power density = 49 mW/cm^2) did not result in any alteration of SCE induction from that caused by chemical treatment alone, even with medium temperature increasing to 39.2°C.

Further experimentation, using the same protocol but with simultaneous exposure to the chemical Adriamycin (Ciaravino et al., 1990) (8) has also shown no alteration of chemically-induced SCE induction as a result of the simultaneous RFR exposure. The results of these experiments are summarized in Table 12, from the manuscript by Ciaravino et al., 1990.

Chromosome Aberration Induction

These investigations are described in a manuscript entitled "Influence of Radiofrequency Radiation on Chromosome Aberrations in CHO Cells and its Interactions with DNA Damaging Agents" (Kerbacher et al., 1990), for 2450 MHz pulsed wave RFR (SAR = 33.8 W/kg, net forward power 600 W) and simultaneous exposure to either mitomycin C (MMC) or Adriamycin (ADM). Treatments with the MMC or ADM were for a total of 2.5 hr, and encompassed the 2-hr RF radiation exposure period. The CHO cells were analyzed for chromosome aberrations using an *in situ* method. Relative to the mitomycin C treatment alone at 37°C, for 2 different concentrations, no alteration was observed in the extent of chromosome aberrations induced by either simultaneous RF radiation exposure or convection heating to equivalent temperatures. At the Adriamycin concentration used, most of the indices of chromosome aberrations which were scored indicated a similar absence of any effect. In one comparison, however, after simultaneous RF radiation and Adriamycin treatment, a small but statistically significant increase was observed for the induced chromosome aberration events per 100 cells compared to chemical exposure alone at 37°C. Since the same small but statistically significant increase was observed in the convection heated temperature control, it would appear that the observed effect could be temperature dependent, and not a phenomenon specific for RF radiation.

3. Hyperthermic potentiation of SCE induction by Adriamycin in CHO cells.

In addition to the experiments described above, an investigation was performed to test the hypothesis that exposure of exponentially growing cells to ADM at an elevated temperature would result in both a greater SCE frequency and cytotoxicity than would be observed in the presence of the drug at 37°C.

TABLE 12. THE FREQUENCY OF SCEs IN CHINESE HAMSTER OVARY CELLS AFTER EXPOSURE TO 2450 MHz RADIOFREQUENCY RADIATION WITH AND WITHOUT ADRIAMYCIN AT CONCENTRATIONS OF 7.75×10^{-6} M

Number of SCEs per cell ± S.E.M.

ADRIAMYCIN (7.75×10^{-7} M)

		1	2	3	4
A.	CONTROL	7.92 ± 0.36	10.12 ± 0.47	9.48 ± 0.40	8.22 ± 0.37
B.	C-ADM	16.90 ± 0.74	17.38 ± 0.83	13.42 ± 0.80	13.86 ± 0.54
C.	TC	8.72 ± 0.38	8.10 ± 0.40	8.38 ± 0.45	8.94 ± 0.43
D.	TC-ADM	20.90 ± 0.83	15.76 ± 0.61	15.14 ± 0.81	13.74 ± 0.70
E.	RF	8.70 ± 0.39	8.90 ± 0.48	9.06 ± 0.43	8.10 ± 0.44
F.	RF-ADM	17.66 ± 0.93	15.94 ± 0.75	16.18 ± 0.74	13.60 ± 0.56

ADRIAMYCIN (7.75×10^{-7} M)

		1	2	3	4
A.	CONTROL	9.48 ± 0.56	8.18 ± 0.45	11.00 ± 0.57	
B.	C-ADM	19.68 ± 0.83	19.62 ± 1.04	23.86 ± 0.93	
C.	TC	8.72 ± 0.42	8.44 ± 0.45	12.38 ± 0.50	
D.	TC-ADM	20.76 ± 1.07	17.04 ± 0.75	28.78 ± 1.15	
E.	RF	8.90 ± 0.41	9.14 ± 0.46	11.20 ± 0.52	
F.	RF-ADM	22.46 ± 1.09	22.06 ± 0.98	23.06 ± 0.93	

The results, appearing in the appended manuscript (Appendix A) entitled "Hyperthermic Potentiation of SCE Induction and Cell Killing by Adriamycin in CHO Cells (Ciaravino and Holahan, in preparation), showed that Adriamycin exposure at 41°C resulted in greater cell killing than exposure to ADM at 37°C. With respect to genotoxicity, SCE frequency was found to be higher after exposure of Adriamycin at 37°C than at 41°C if cells were harvested at 24 hr, but if the harvest time was delayed for 6 hr, the SCE frequency was greater after ADM exposure at 41°C than at 37°C.

CONCLUSIONS

RFR Alone, and Simultaneous RFR and Chemical Exposures

In conclusion, from the reports described, RFR at low and moderate SARs and power densities, at the frequencies indicated, does not show any genotoxic activity by itself. In addition, there is no evidence that such exposures at 2450 MHz result in any increased risk, or in fact any alteration, of genotoxicity resulting from a simultaneous exposure to genetically hazardous chemicals. This conclusion is with the proviso that the temperature during the exposure period did not exceed 40°C. Temperatures above this are also not likely to be the case in human occupational or public exposures, with the exception of the specific medical application of combined modality cancer therapy; these experiments therefore indicate a safety level of RFR exposure when concern is for genotoxic effects.

RFR and Ionizing Radiation Exposure

While some of the studies described above focused on the potential interactions between RFR and chemicals, there is also cause for concern for RFR interactions with ionizing radiation, since the latter is known to cause cancer in humans, and is a potential human mutagen. The investigation of this possibility includes studies of effects on human cells, as well as on rodent cells, and the development of techniques and the availability of facilities to perform simultaneous ionizing radiation and microwave exposures. Because of an extended delay in the delivery of a specially designed and constructed dual frequency microwave transmitter to the UTHSCSA, it was unnecessary to develop an alternative in vitro exposure system for use in such studies. As described in this report, a novel approach was developed where a chemical containing ^{32}P, a β^- emitting radioactive isotope, was mixed into a lower agar layer in a T-25 flask, and then agar walls were erected on top of this lower layer to create a "well" or "chamber." This well, into which mammalian cells in suspension could be injected, served as an exposure chamber. An advantage of this agar-well exposure system is its potential for use with the air temperature controlled incubator system first designed by investigators at the U.S. Air Force School of Aerospace Medicine (for circular waveguide exposures); the technique is being modified to allow vertical downward exposures of cells in the flasks to microwave radiation in the far field (of an antenna horn).

The method for creating such a ^{32}P agar well exposure system has been described herein. Also described were its successful use in the induction of ^{32}P β- particle toxicity, as well as induction of mutations at the XPRT locus in AS52 Chinese hamster cells by ^{32}P β- particles. Counterbalancing this successful demonstration of radiation-induced mutagenic action in the

agar well system was a separate experimental determination of an undesirable nature. We report here that the survival of AS52 cells incubated at elevated temperatures in the agar wells (without any radioactivity) was consistently lower than the survival of cells exposed to hyperthermia as a surface attached population. This agar heating effect is cause for concern in the potential use of the agar well system for simultaneous microwave and ^{32}P exposure, since the RFR can be expected to increase the temperature. The system can still be applied if the temperature can be maintained at 37°C using the air incubator system, and where temperature monitoring is carefully and correctly performed.

BIBLIOGRAPHY

Meltz ML, Walker KA, and Erwin DN (1987). Radiofrequency (Microwave) Radiation Exposure of Mammalian Cells During UV-Induced DNA Repair Synthesis. Radiation Res 110:255-266.

Meltz ML, Eagan P, and Erwin DN (1989). Absence of Mutagenic Interaction Between Microwaves and Mitomycin C in Mammalian Cells. Environmental and Molecular Mutagenesis 13:294-303.

Ciaravino V, Meltz ML, and Erwin DN (1987). Effects of Radiofrequency Radiation and Simultaneous Exposure with Mitomycin C on the Frequency of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells. Environmental Mutagenesis 9:393-399.

Kerbacher JJ, Meltz ML, and Erwin DN (1990). Influence of Radiofrequency Radiation on Chromosome Aberrations in CHO Cells and its Interaction with DNA-Damaging Agents. Radiation Res 123:311-319.

Meltz ML, Eagan P, Harris CR, and Erwin DN (1988). Dosimetry Considerations in Far Field Microwave Exposure of Mammalian Cells. Physiol Chem and Physics and Medical NMR 20:23-30.

Meltz ML, Eagan P, and Erwin DN (1990). Proflavin and Microwave Radiation: Absence of a Mutagenic Interaction. Bioelectromagnetics 11:149-157.

Ciaravino V, Meltz ML, and Erwin DN (1990). Absence of a Synergistic Effect Between Moderate Power Radiofrequency Radiation and Adriamycin on Cell Cycle Progression and Sister Chromatid Exchange. In Preparation.

APPENDIX A

HYPERTHERMIC POTENTIATION OF SCE INDUCTION AND CELL KILLING BY ADRIAMYCIN IN CHO CELLS

Victor Ciaravino and Patricia K. Holahan

Department of Radiology

The University of Texas Health Science Center

San Antonio, TX 78284-7800

ABSTRACT

Chinese hamster ovary (CHO) cells have been exposed to hyperthermic treatment (41°C) in combination with adriamycin (ADM). The study was designed to test the hypothesis that exposure of exponentially growing cells to ADM at an elevated temperature would result in both a greater SCE frequency and cytotoxicity than would be observed in the presence of the drug at 37°C. In addition to these endpoints, the increase in cellular multiplicity was examined as a measure of division delay and an analysis of first, second, and third division metaphases was assessed for cell cycle effects. The results showed that simultaneous ADM and 41°C treatments resulted in greater cell killing than exposure to ADM at 37°C. With respect to genotoxicity, SCE frequency was found to be higher in exposures of ADM at 37°C if cells were harvested at 24 hr but if harvest time was delayed for 6 hr, SCE frequency was greater at 41°C in the presence of ADM. From the multiplicity determinations and metaphase analysis, it was evident that there was a greater division delay if cells were exposed to ADM at 41°C than 37°C.

INTRODUCTION

The cytoxic and genotoxic effects of an antineoplastic such as adriamycin (ADM) have been well documented (Barranco, 1975; Belli, 1979; Carter, 1975; Harris et al., 1979). ADM intercalates between adjacent base pairs in DNA (Zunino et al., 1972) and as a result, interferes with normal DNA synthesis. This interaction with the DNA of cells can ultimately lead to genetic consequences as well as cytotoxicity. One measurement of a genetic endpoint of chemicals is by sister chromatid exchange (SCE) analysis. The widespread use and study of SCE is very much related to their apparent association with mutagenic and carcinogenic phenomena (Perry and Evans, 1975); thus SCE are routinely used as a screening tool for mutagen testing. Although the molecular mechanisms for the formation of SCE are not known specifically, several possible mechanisms have been discussed by Latt et al. (1984).

There is a vast amount of existing knowledge concerning the induction and formation of SCEs and their relationship to other biological endpoints, such as carcinogenesis, mutagenesis, transformation, clastogenesis, DNA damage and repair, and cellular toxicity (Tice, 1984). In addition, the formation of SCEs is influenced by a variety of factors that can be controlled in vitro. These factors include bromodeoxyuridine (BrdUrd) concentration (Cerni, 1984), serum (Kato and Sandberg, 1977), and light (Ikushima and Wolff, 1974; Monticone and Schneider, 1979). One other factor of concern is the influence of temperature on SCE formation. This topic has been represented in the literature using a variety of scientific protocols and cell types and has been summarized in Table A-1. The basic trends of these studies indicate that SCE frequencies were temperature dependent when cells were incubated through 2 or more cell cycles at temperatures >37°C (up to 42°C). Short-term exposures (several hours) only yielded a higher SCE frequency in one study (Livingston and Dethlefsen, 1979). Furthermore, exposure temperatures <37°C also resulted in increased SCE frequencies. The mechanism explained for these results relates to depressions in DNA synthesis and a subsequent lengthening of cell cycle time.

Three of the studies in Table A-1 not only measure SCE frequency as it correlates to hyperthermic conditions, but they also assess potentiation of a drug treatment in combination with hyperthermia. Miura et al. (1986) pulse-treated

TABLE A-1. THE EFFECT OF TEMPERATURE ON SCE RESPONSE IN HUMAN LYMPHOCYTES AND RODENT CELL LINES

System	Temperature (°C); Duration or Treatment	Response of SCE Frequency Compared to 37° Incubations (-) reduction; (0) no effect; (+) increase	Reference
Human lymphocytes and Indian muntjac lymphocytes	33-42; 56 and 72 hr	33°C (-) 40°C (+) 42°C (+)	Das and Sharma, 1984
Human lymphocytes Chinese hamster K-1	43 and 45, 2-4 hr	43°C (0) 45°C (0)	Vig et al., 1982
Human lymphocytes	35 and 40, 72 hr	35°C (+) 40°C (+)	Pandita, 1983
Human lymphocytes	0-41, 2 hr	full range (0)	Miura et al., 1986
Human lymphocytes	35 and 39, 72 hr	35°C (+) 39°C (+)	Abdel-Fadil, 1982
V-79	33 and 40, 27 hr	33°C (0) 40°C (+)	Speit, 1980
FR3T3 rat cell line	33 and 40.5, 5 days	33°C (0) 40.5°C (0)	Cerni, 1984
D-6 Chinese hamster	31-42; 24 hr	>39°C (+) <39°C (0)	Kato, 1980
CHO	44; 20, 40 and 60 min	44°C (+)	Livingston and Dethlefsen, 1979

human lymphocytes in G_0 with mitomycin C (MMC), ethyl methanesulfonate (EMS), or 4-nitroquinoline N-oxide (4NQO) at various temperatures ranging from 0°C to 41°C and then cultured at 37°C. The results showed that in MMC- or EMS-exposed cultures the SCE frequency increased continuously w.th increasing treatment temperature; SCE frequencies in cells exposed to 4NQO showed no temperature-dependent changes. Vig et al. (1982) studied the effects of hyperthermia as a potentiator of SCE induction by the anticancer antibiotics ADM, MMC, and bleomycin. The data showed that there was an increased potential of the chemicals to induce chromosome aberrations when applied at temperatures higher than 37°C; however, no potentiating effect was observed on the induction of SCEs. Furthermore, Kaul (1985) also found that treatment of human lymphocytes in vitro with 1,3- propane sultone (PS), a monofunctional alkylating agent, resulted in a higher frequency of chromosome aberrations when treated at 45°C than at 37°C. However, this study did not examine the hyperthermic enhancement of SCE induction in cells exposed to PS.

Hyperthermia in combination with radiation or antineoplastic antibiotics, has potential use in cancer therapy (Raymond et al., 1980). Several studies on in vitro systems have shown that there was a thermal enhancement of ADM-induced cytotoxicity (for review see Streffer, 1987). For example, EMT-6 cells were exposed to 0.5 μg/ml ADM at 37°, 41°, and 42°C for up to 6 hr and a synergistic effect was observed at 42°C when cell survival was the endpoint.

The present study has focused on exposures of Chinese hamster ovary (CHO) cells to hyperthermic treatment (41°C) in combination with adriamycin (ADM). The study has been designed to test the hypothesis that exposure of exponentially growing cells to ADM at an elevated temperature will result in both a greater SCE frequency and cytotoxicity than would be observed in the presence of drug at 37°C. Further experiments were conducted to examine possible mechanism(s) responsible for the induced effect. These experiments include the assessment of colony multiplicity for division delay and an analysis of first, second, and third division metaphases for cell cycle effects.

MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells were maintained in 75 cm^2 tissue culture flasks in Ham's F-10 medium supplemented with 10% fetal bovine serum and gentamycin (40 μg/ml). Exponentially growing cultures were incubated at 37°C in a 95% air/5% humidified incubator.

Hyperthermia/Drug Treatments

For determination of either survival or SCE formation, monolayer cultures of cells were resuspended with 0.25% trypsin 24 hr prior to treatment. Cells were seeded at a density of 2×10^5 cells/flask in 25 cm^2 flasks containing a final total volume of 8 ml medium and incubated for 24 hr at 37°C to allow for attachment and proliferative growth.

Immediately prior to treatment, adriamycin (Adria Laboratories, Columbus, OH) was added to the flasks for drug exposure (final concentration 0.5 μg/ml) and placed in a 37°C water bath for 5 min before heat treatment. To prevent leakage, the caps of all flasks were sealed with Parafilm before immersing into water baths. Flasks that received no drug were also sealed and placed in a 37°C water bath. Hyperthermic treatments were conducted by total immersion of the flasks into a 41° (± 0.05°C) water bath for up to 6 hr. After heating, the flasks

were returned to 37°C for 5 min to ensure constant cool-down kinetics. Exposures were conducted under yellow lights to prevent photolysis of ADM. Cells were either plated for survival or incubated for SCE analysis as described below. Cells were exposed to the drug at either 37° or 41°C as well as 41°C without ADM. Flasks were exposed in duplicate.

SCE Analysis

For SCE analysis, bromodeoxyuridine (BrdUrd; Sigma, St. Louis, MO) was added (20 μM) to each of the flasks immediately prior to treatment. Following treatment, medium in all flasks was removed and each flask was rinsed with several ml fresh medium. The flasks were then filled with 8 ml prewarmed (37°C) fresh medium containing 20 μM BrdUrd and incubated at 37°C in the dark for 24 hr. At 21-hr posttreatment, 0.2 ml colcemid (GIBCO) (10 μg/ml) was added to each culture flask. Three hours later, gentle shaking was used to detach mitotic cells, and the medium containing these cells was removed. In a final experiment, the time of harvest was varied to 24-, 27- and 30-hr postexposure. Slides of the mitotic cell suspensions were then prepared and stained by the Fluorescence plus Giemsa (FPG) technique of Perry and Wolff (1974). The slides were stained in a Hoechst 33258 solution (5 μg/ml), pH7) for 20 min, mounted in phosphate buffer, exposed to blacklight (GE F15T8BLB,15W) for 10 min at a distance of 2 cm, and then stained with a 4% Giemsa solution at pH7 for 5 min. The slides were then coded and the frequency of SCEs for each cell was scored. Thirty cells were scored for each treatment group. In order to assess treatment effects on cell progression, the ratios of first, second and third division cells were determined by scoring 100 cells from each treatment group (Tice et al., 1976; Morimoto and Wolff, 1982). The data were analyzed with a two-way analysis of variance (ANOVA) test. Additionally, where differences appeared between treatment groups, Student t-tests were conducted.

Determination of Cell Survival

For survival studies, cells were trypsinized and resuspended by pipette immediately following treatment. Cells were counted using a Coulter counter (Coulter Electronics, Hialeah, FL), diluted, and plated into replicate 25 cm^2 flasks for colony formation. Flasks were then incubated at 37°C for 7 days and macroscopic colonies were fixed, stained, and counted to determine surviving fraction.

Multiplicity Determination

To determine whether treatment delayed cell division, 1 experiment was conducted to measure the increase in cellular multiplicity with time after treatment. For this experiment, 5 x 10^3 cells were plated into 25 cm^2 flasks 16 hr prior to treatment. The treatments were the same as for SCE and survival analysis. At various times, up to 30 hr following treatment, cells (duplicate flasks for each treatment group) were fixed with 3:1 methanol:glacial acetic acid. The flasks were then stained with 5% Giemsa for 5 min and the cellular multiplicity was calculated as the number of cells per colony forming unit. One hundred colony forming units were scored for each flask.

RESULTS

Survival of CHO cells following exposure to 0.5 μg/ml ADM at 37°C or 41°C is shown in Figure A-1. There is no difference in the amount of cell killing by 41°C

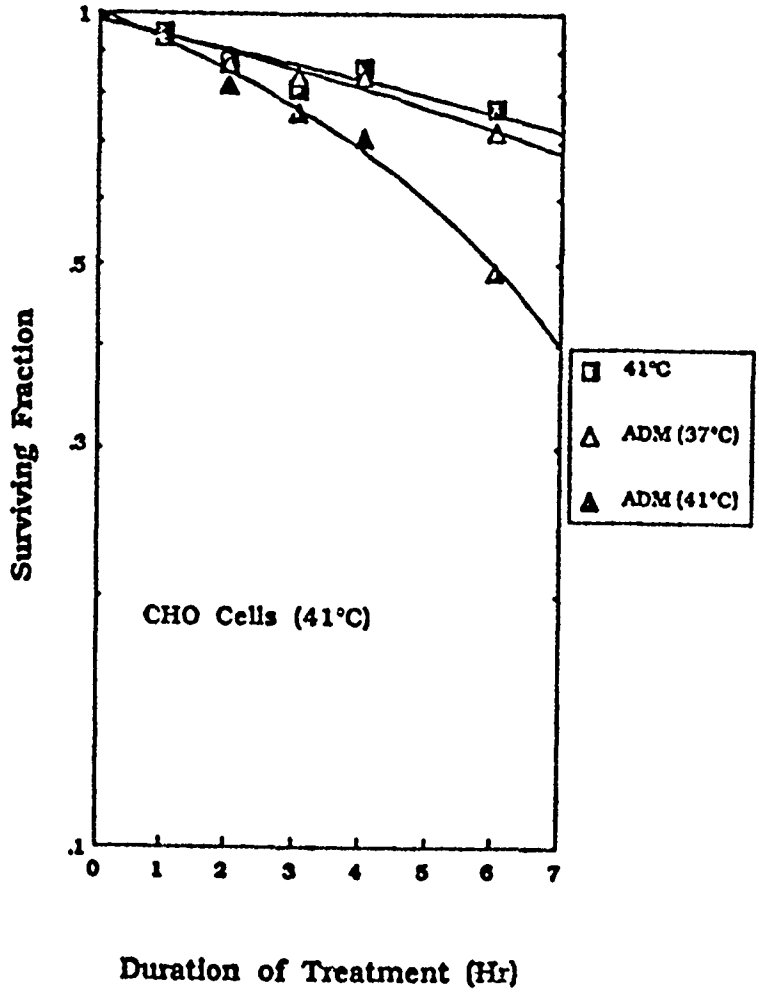

Figure A-1. Survival of CHO cells following exposure to 0.5 µg/ml ADM at 37 or 41°C. Cells were exposed to ADM only during the heat treatment. Data are the average of 3 individual experiments.

or ADM (37°C) alone, but there is an increase in ADM cytotoxicity at 41°C. The same treatments were used for SCE assessment and the results are depicted in Figure A-2. Hyperthermic treatment of 41°C for up to 6 hr does not appear to increase the frequency of SCE relative to the unheated control population. ADM, with or without heat treatment, increases SCE induction above control levels, albeit a differential response is evident.

At the earliest treatment times (1 and 2 hr), ADM (41°C) results in a slightly higher frequency of SCEs than ADM (37°C). There appears to be a crossover between 2 and 3 hr, such that by 4 and 6 hr ADM (37°C) is more effective at producing SCEs than ADM (41°C) ($p<0.05$). This was somewhat contrary to the cell survival data which showed enhanced cell killing by ADM at 41°C with increasing time of exposure (Fig. A-1). Furthermore, there appeared to be fewer second division cells in the population exposed to ADM for longer times. This is illustrated in Figure A-3 which shows the percentage of second division cells as a function of duration of treatment. Hyperthermia alone did not appear to delay progression of cells into second division. There was a delay for ADM (37°C) treated cells such that 24 hr after a 6-hr exposure only 78% of the mitotic figures were second division cells, representing a division delay. Furthermore, after 6 hr of ADM treatment at 41°C, only 25% of the cells were in second division, indicating a greater effect on cell cycle.

We further studied this progression delay as a possible mechanism for the lower frequency of SCE after 6-hr ADM exposure at 41°C. At this temperature and ADM concentration, cell survival was effected much more than an exposure of ADM at 37°C. To examine whether cells that were delayed longer had the same frequency of SCEs as those harvested at 24 hr, we conducted another experiment using the same treatment parameters but harvesting the cells for SCE formation at longer times. Figure A-4 shows SCE frequencies as a function of duration of treatment for 24-, 27-, and 30-hr harvest times. Figure A-4a shows that the response of SCE frequency with respect to treatment duration follows a similar response to that observed in Figure A-2. Although in this experiment the crossover of the 2 curves [ADM (37°C) vs. ADM (41°C)] occurred after 4 hr of treatment, a 6-hr ADM (41°C) showed slightly lower SCEs than the ADM (37°C) point. At 27 hr (Figure A-4b), the responses of the 2 treatments are very similar, while Figure A-4c illustrates that at a 30-hr harvest time the ADM (41°C) treatment has a higher SCE frequency than the ADM (37°C). Table A-2 further illustrates the effect of hyperthermia, with or without ADM, on cell progression by showing the percentage of first, second, and third division cells after treatment. The data shown are for 3 harvest times of 24-, 27-, and 30-hr posttreatment. Cells exposed to ADM at 37°C for 6 hr showed about 25% first division cells at 24 hr but nearly all second division cells by 27 hr posttreatment. However for a 6 hr ADM exposure at 41°C, 15% of the cells had not progressed past first division by 30 hr after treatment. At the same time, untreated (control) cells were already progressing through a third mitosis (23%) by the 30 hr harvest.

Progression delay was assessed in which an experiment measured the increase in cellular multiplicity with time after treatment. As seen with this delay into second division, cells that are heated with adriamycin at 41°C showed a slower increase in cell number (Fig. A-5). The greatest prolongation of cell progression was seen for cells treated for 6 hr. Six hours of ADM exposure at 37°C also slowed cells. From the slope of the multiplicity curve, an approximate doubling time was calculated for each treatment (Table A-3). Whereas there was no effect of 41°C alone, the doubling time increased with longer exposures to ADM at both exposure temperatures.

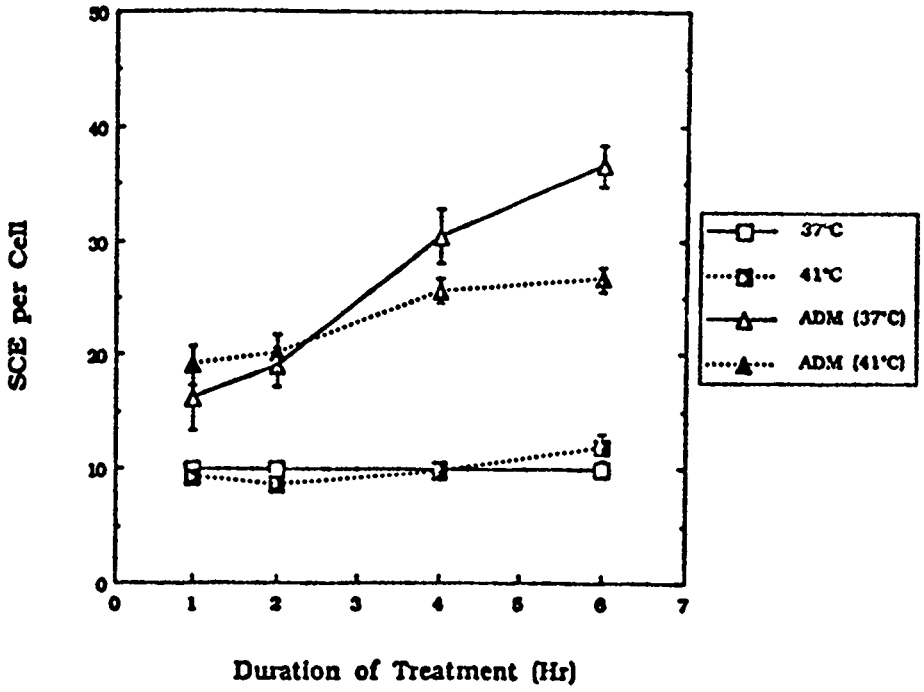

Figure A-2. Frequency of sister chromatid exchanges (SCE) in CHO cells exposed to 0.5 µg/ml ADM at 37 or 41°C. Cells were harvested 24 hr following treatment. Data are average of 6 individual experiments.

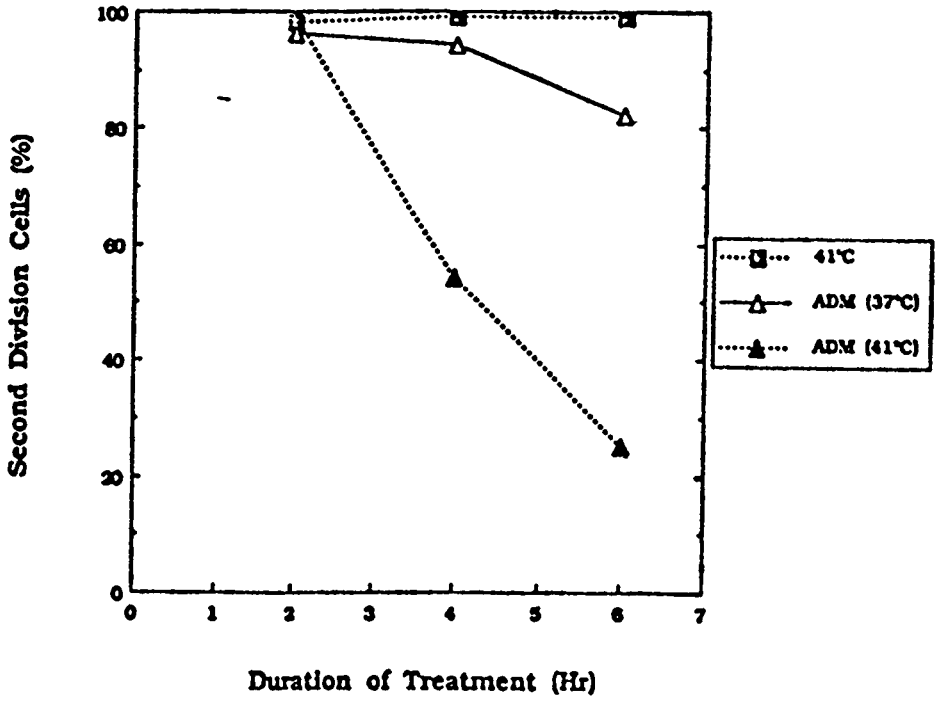

Figure A-3. Effect of heat and adriamycin on cell progression. CHO cells were exposed to 0.5 µg/ml ADM at 37 or 41 °C and mitotic cells harvested 24 hr later. The percentage of total cells that progressed into a second mitosis (second division cells) was determined for each treatment. Data are from a representative experiment.

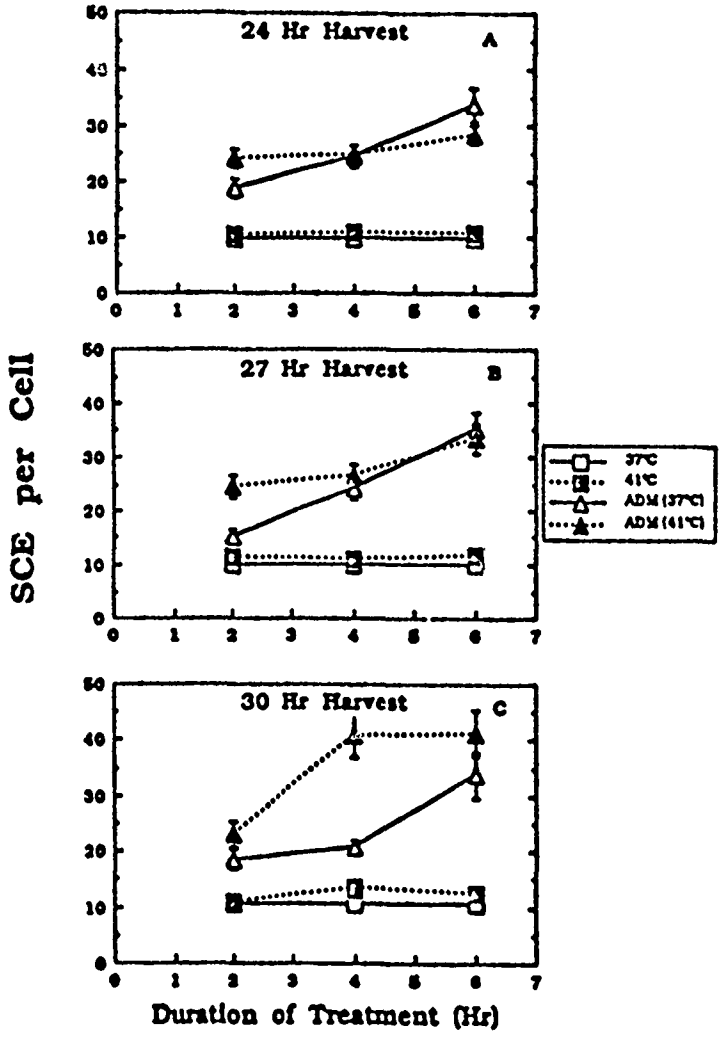

Figure A-4. Frequency of SCE in CHO cells exposed to 0.5 µg/ml ADM at 37 or 41°C harvested at 24-, 27-, or 30-hr following treatment. Data are from 1 experiment.

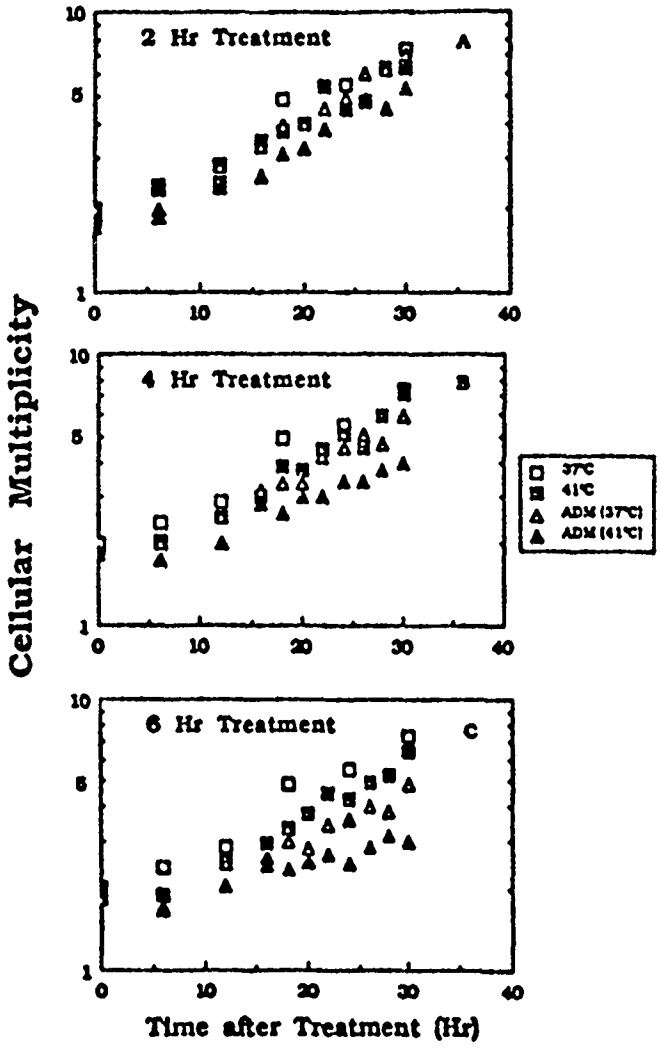

Figure A-5. Cellular multiplicity for CHO cells exposed to 0.5 µg/ml ADM at 37 or 41°C as a function of time following treatment. Cells were treated for 2 (panel A), 4 (panel B), or 6 (panel C) hr. Cells were exposed to ADM only during the heat treatment.

TABLE A-2. PERCENT FIRST, SECOND, AND THIRD DIVISION CHO CELLS AFTER TREATMENTS OF ADM AT 37°C AND 41°C AS WELL AS TREATMENT ALONE AT 41°C. DATA ARE FOR HARVEST TIMES OF 24-, 27-, AND 30-HR POST TREATMENT

		Time of Harvest (Hr)								
		24			27			30		
Treatment	Duration	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Control	---	0	100	0	0	90	10	0	77	23
41°	2	0	100	0	0	100	0	0	83	17
	4	0	100	0	0	99	1	0	93	7
	6	2	98	0	0	95	5	1	85	14
37°ADM	2	3	97	0	0	100	0	0	93	7
	4	8	92	0	7	93	0	1	99	0
	6	24	76	0	2	98	0	5	95	0
41°ADM	2	9	91	0	3	97	0	2	98	0
	4	30	70	0	10	90	0	10	90	0
	6	70	30	0	26	74	0	15	85	0

TABLE A-3. DOUBLING TIME FOR CHO CELLS FOLLOWING TREATMENT WITH ADM AT 37°C OR 41°C. DOUBLING TIME WAS CALCULATED FROM SLOPE OF CELLULAR MULTIPLICITY DATA (FIGURE A-5).

TREATMENT	DOUBLING TIME
Control	14.0
41°C - 2 hr	14.3
41°C - 4 hr	13.0
41°C - 6 hr	14.1
ADM(37°C) - 2 hr	13.3
ADM(37°C) - 4 hr	15.3
ADM(37°C) - 6 hr	18.9
ADM(41°C) - 2 hr	14.6
ADM(41°C) - 4 hr	20.4
ADM(41°C) - 6 hr	33.1

DISCUSSION

Thermal enhancement of anticancer antibiotics such as adriamycin has been studied for the last decade. Hahn et al. (1975) exposed exponentially growing EMT-6 cells to 0.5 μg/ml ADM at 37°, 41° and 42°C for up to 6 hr. At 41°C there was only a small enhancement of the effect on cell survival by the combination of the heat and chemicals, similar to our results in this study. At 42°C, however, there was a striking synergism. Thermal enhancement has since been studied and confirmed by others (Streffer, 1987). The studies have involved many different cell lines and a wide range of temperatures, with the majority focusing on cell survival as their endpoint. Vig et al. (1982) reported on the hyperthermic potentiation of chromosome aberrations by 3 anticancer antibiotics; adriamycin, mitomycin C, and bleomycin. The data showed that there was increased potential of these chemicals to induce chromosome aberrations in human lymphocytes and Chinese hamster K-1 cells when applied at temperatures above 37°C (43°C). The authors felt that the potentiation was due either to true synergism in the case of bleomycin, or facilitation of entry of larger quantities of the drug, as in the case of adriamycin, into the cells. This agrees with fluorescence studies measuring cellular drug uptake in EMT-6 cells (Hahn et al., 1975). The authors noted that no potentiating effect was observed on the induction of SCEs.

The effect of temperature on SCE response has been studied by a number of investigators and is summarized in Table A-1. Das and Sharma (1984) studied the incidence of SCEs and cell proliferation kinetics in peripheral blood lymphocytes of humans and muntjac grown at 33 to 42°C. The frequency of SCEs increased as a function of growth temperature. At a given temperature, however, the frequency of SCEs varied with sampling times; cells sampled at earlier times showed fewer SCEs than did those harvested late.

The results of Das and Sharma (1984) were closely related to what was observed in the present study, one difference being that ADM was used in conjunction with hyperthermia. The data shown in Figure A-1 show that an increase in ADM cytotoxicity exists for cells exposed at 41°C over that which is observed at 37°C or at 41°C without the chemical. The interaction between heat and ADM is either independent or additive in nature, but does not appear to be synergistic with this combination of temperature and drug concentration. These results are similar to those observed by Vig et al. (1982) who postulated that with the higher temperature, larger quantities of the drug penetrated into the cells. When we observed SCE frequencies at the same treatment regimens it was found that the ADM (41°C) exposure did not produce higher levels of SCE than the ADM (37°C) treatment. After an analysis of the cells for proliferation kinetics it was observed that cells exposed to the ADM (41°C) treatment only contained 25% second division cells 24-hr following treatment, indicating a cell cycle delay. This implied that we may be examining a selective pool of second division cells and thus at the 24 hr harvest time, a small "window" of cycling cells is being scored. At the time of exposure SCE induction may have been greater at a specific phase of the cell cycle that exhibits a greater delay. Only those cells cycling at a control rate (~14 hr) are being scored. Thus, although these "slower" cells may contain a higher SCE frequency, it will be undetectable at a 24-hr harvest time. Therefore by conducting an experiment whereby harvest times were expanded to 27 and 30 hr, we could now test this hypothesis and observe SCE frequencies at these later times.

Figure A-4 shows SCE frequencies as a function of duration of treatment for 24-, 27-, and 30-hr harvest times. At a 30-hr harvest the 4 and 6-hr treatments for ADM (41°C) were significantly higher ($p<0.05$) than ADM (37°C) indicating that the "slower" cells have now had an additional 6 hr to reach a second mitosis in the harvesting "window" and these cells were increasingly perturbed with respect to SCE formation. This agrees with the data of Das and Sharma (1984) that showed higher SCE in heated cells at later harvest times.

Ishii and Watatani (1984) concluded that the process of SCE formation occurred during the S phase of the cell cycle and this conclusion is supported by others (Ishii and Bender, 1980; Kato, 1977, 1977b). In the present experiments, the results suggest that not only do the S phase cells contain an increased frequency of SCEs, these same cells take longer to traverse the cell cycle. Table A-2 further illustrates the effect of hyperthermia, with or without ADM on cell progression by showing the percentage of first, second, and third division cells after treatment. When comparing the 37°C and 41°C ADM 6-hr treatments, one can see a lower percentage of second division cells in the latter group 24 hr after exposure. At the 27-hr harvest there is still a relatively low percentage of second division cells in the 41°C ADM (6 hr treatment) population. While the frequencies of second division cells in the two treatment groups do approach one another at 30 hr, there still is a substantial difference indicating progression is affected.

This progression delay was verified by conducting a cellular multiplicity experiment. The results in Figure A-5 show that cells heated with ADM at 41°C display a slower increase in cell number than other treatments. The prolongation of cell progression correlated well with the duration of treatment. This is evidenced by the calculation of doubling time from the slope of the multiplicity curve (Table A-3). It is evident that doubling time increased with duration of treatment. Additionally, for a 6-hr treatment, doubling time increased from a 41°C treatment (14.1 hr) to 18.9 hr for an ADM (37°C) treatment. This increased further to 33.1 hr when exposing to ADM at 41°C. It should be mentioned that these doubling times are calculated for total cellular multiplicity and not the multiplicity of viable cells such that the doubling time of viable cells may be somewhat shorter than the calculated value. However, it is evident that there is a lengthening of cell cycle time for cells heated at 41°C for 4 or 6 hr in the presence of ADM.

In concluding, it was found that simultaneous ADM and 41°C treatments affected CHO cells such that a 6-hr treatment had the greatest effects on cell survival. With respect to genotoxicity, it was found that SCE frequency was higher in exposures of ADM at 41°C, but this was not noticeable unless later harvest times were observed. Thus, in order to fully assess the effect of hyperthermia and chemicals on SCE frequencies, future research should determine when the peak of second division cells is harvested and, at that time, determine SCE response.

ACKNOWLEDGMENTS

This research was supported by NIH BRSG 2507RR-05654-19 and USAF Contract #F33615-87-C-0610. The authors would like to acknowledge the technical assistance of Ms. Phyllis Eagan and Pat Fernandez. We would also like to thank Ms. Joanne Murray for help in preparation of this manuscript.

REFERENCES

Abdel-Fadil, M.R., Palmer, C.G., and Heerema, N., 1982, Effect of temperature variation on sister-chromatid exchange and cell-cycle duration in cultured human lymphocytes. Mutation Research 104:267-273.

Barranco, S.C., 1975, Review of the survival and cell kinetic effects of adriamycin (NSC-123127) on mammalian cells. Cancer Chemotherapy Reprints 6:147.

Belli, J.A., 1979, Radiation response and adriamycin resistance in mammalian cells in culture. Frontiers in Radiation Therapy and Oncology 13:9.

Carter, S.K., 1975, Adriamycin: A review. Journal of the National Cancer Institute 55:1265.

Cerni, C., 1984, Effect of tissue culture variables on sister chromatid exchange in a nontransformed rat cell line. In Vitro 20:305-313.

Das, B.C., and Sharma, T., 1984, Effects of temperature on the frequency of sister chromatid exchanges (SCEs) in peripheral blood lymphocytes of man and muntjac. Environmental Mutagenesis 6:25-31.

Engelhardt, R., 1987, Hyperthermia and drugs. Recent results in Cancer Research, 104:136-203.

Hahn, G.M., Braun, J., and Har-Kedar, I., 1975, Thermochemotherapy: synergism between hyperthermia (42-43°) and adriamycin (or bleomycin) in mammalian cell inactivation (cancer chemotherapy/cell membranes). Proceedings of the National Academy of Sciences USA, 72:937-940.

Harris, J.R., Timberlake, N., Henson, P., Schinke, P., and Belli, J.A., 1979, Adriamycin uptake in V79 and adriamycin-resistant Chinese hamster cells. International Journal of Radiation Oncology and Biological Physics 5:1235.

Ikushima, T., and Wolff, S., 1974, Sister chromatid exchanges induced by light flashes to 5-bromodeoxyuridine and 5-iododeoxyuridine substituted Chinese hamster chromosomes. Experimental Cell Research 87:15-19.

Ishii, Y., and Bender, M.A., 1980, Effects of inhibitors of DNA synthesis on spontaneous and ultraviolet light-induced sister-chromatid exchanges in Chinese hamster cells. Mutation Research 79:19-32.

Ishii, Y., and Watatani, M., 1984, Cell-stage dependence of the formation of SCEs and chromosomal aberrations. Sister Chromatid Exchanges, Part A, R.R. Tice and A. Hollaender (eds.), New York: Plenum Press, pp.173-180.

Kato, H., 1977, Mechanisms for sister chromatid exchanges and their relation to the production of chromosomal aberrations. Chromosoma 59:179-191.

Kato, H., 1977, Spontaneous and induced sister chromatid exchanges as revealed by a BUdR-labeling method. International Review of Cytology 49:55-97.

Kato, H., 1980, Temperature-dependence of sister chromatid exchange: An implication for its mechanism. Cancer Genetic and Cytogenetics 2:61-67.

Kato, H., and Sandberg, A.A., 1977, The effect of sera on sister chromatid exchanges in vitro. Experimental Cell Research 109:445-448.

Kaul, B.L., 1985, Induction of chromosomal aberrations and sister chromatid exchanges by propane sultone in human lymphocytes in vitro. Toxicology Letters 28:139-142.

Latt, S.A., Schreck, R.R., D'Andrea, A., Kaiser, T.N., Schlesinger, F., Lester, S., and Sakai, K., 1984, Detection, significance and mechanism of sister chromatid exchange formation: Past experiments, current concepts, future challenges. Sister Chromatid Exchanges, Part A, R.R. Tice and A. Hollaender (eds.), New York: Plenum Press, pp 11-40.

Livingston, G.K., and Dethlefsen, L.A., 1979, Effects of hyperthermia and X-irradiation on sister chromatid exchange (SCE) frequency in Chinese hamster ovary (CHO) cells. Radiation Research 77:512-520.

Miura, K., Morimoto, K., and Koizumi, A., 1986, Effects of temperature on chemically induced sister-chromatid exchange in human lymphocytes. Mutation Research 174:15-20.

Monticone, R.E., and Schneider, E.L., 1979, Induction of sister chromatid exchanges in human cells by fluorescent light. Mutation Research 59:215-221.

Morimoto, K., and Wolff, S., 1982, Cell cycle kinetics in human lymphocyte culture. Nature 288:604-606.

Pandita, T.K., 1983, Effect of temperature variation on sister chromatid exchange frequency in cultured human lymphocytes. Human Genetics 63:189-190.

Perry, P., and Evans, H.S., 1975, Cytological detection of mutagen-carcinogen exposures by sister chromatid exchange. Nature 258:121-125.

Perry, P., and Wolff, S., 1974, New Giemsa method for the differential staining of sister chromatids. Nature 251:156-158.

Raymond, U., Noell, K.T., Woodward, K.I., Worde, B.I., Fishburn, R.I., and Miller, L.S., 1980, Microwave-induced local hyperthermia in combination with radiotherapy of human malignant tumors. Cancer 45:638-646.

Speit, G., 1980, Effects of temperature on sister chromatid exchange. Human Genetics 55:333-336.

Tice, R., Schneider, E.L., and Rary, J.M., 1976, The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics. Experimental Cell Research 102:232-236.

Tice, R.R., and Hollaender, A. (eds), 1984, Sister Chromatid Exchanges, 25 years of experimental research. Part A: The nature of SCEs, Part B: Genetic Toxicology and Human Studies, New York: Plenum Press.

Vig, B.K., Cornforth, M., and Farook, S.A.F., 1982, Hyperthermic potentiation of chromosome aberrations by anticancer antibiotics. Cytogenetics and Cell Genetics 33:35-41.

Zunino, F., Gambetta, R., DiMarco, A., and Zaccara, A., 1972, Interaction of daunomycin and its derivatives with DNA. Biochimica Biophysica Acta 277:489.

GLOSSARY

BBL - Agar, granulated, from BBL Microbiology Systems

CE - cloning efficiency: the ratio of the number of colonies appearing in a flask, after a defined number of days of incubation, to the number of cells initially seeded in the flask. The latter would be corrected for multiplicity, if this is stated. A colony must have more than a preselected number of cells (e.g., 100 cells) to be counted.

DNA - deoxyribonucleic acid - the genetic material of the cell.

EMS - ethyl methane sulfonate - a known chemical mutagen and monofunctional alkylating agent.

FBS - fetal bovine serum (added at specified concentrations to cell culture medium).

F12CM5 - Ham's F12 medium (complete medium) with 5% fetal calf serum.

F12CM10 - same as above, with 10% fetal calf serum.

DS - refers to medium prepared with dialyzed serum.

Fraction of clonable cells (Day 7) - represents the ratio of the cloning efficiency of the treated cell population to that of the 37°C control cells, when the cells are seeded into soft agar on Day 7 posttreatment.

G1 - the period of the mammalian cell cycle between mitosis and the onset of DNA synthesis.

G2/M - the combined G2 (post-DNA synthetic) and M (mitotic) stages of the mammalian cell cycle.

Gy/min (gray/minute) - physical absorbed dose rate for ionizing radiation.

HI (heat inactivated) - applies to serum which is incubated at 57°C - 60°C for 12 - 45 min.

Initial cloning efficiency (Day 1) - this value represents the cloning efficiency of the treated or control cell population, on the first day after treatment. It is used to calculate the initial surviving fraction (as a result of the treatment).

μCi/ml - microcurie per milliliter.

MPA medium - Ham's F12 medium with 10% fetal calf serum, to which the chemicals xanthine, aminopterin, mycophenolic acid, adenine, and thymidine are added to kill any pre-existing mutants lacking the enzyme hypoxanthine-guanine phosphoribosyl transferase.

Multiplicity flasks: flasks seeded with 5000 (supposedly) single cells which are fixed at the time of treatment. These are counted for determination of the ratio of singlet cells to doublet and triplet cell clusters. This data allows for the calculation of the actual number of colony forming units seeded for cloning efficiency assay.

NP-40 NONIDET P-40 - a nonionic detergent (from SIGMA Chemical Co.).

RFR - radiofrequency radiation.

RNAse - ribonuclease enzyme, used to digest (break down) ribonucleic acid.

S - the DNA synthetic period of the mammalian cell cycle.

SCE - sister chromatid exchange, the exchange of sections of adjoining arms of a chromosome.

Surviving fraction - represents the cell viability (reproductive integrity) after treatment. It is the ratio of the initial (Day 1) cloning efficiency of treated cells to the initial (Day 1) cloning efficiency of 37°C control cells.

^{32}P - phosphorus-32, radioactive isotope emitting β^- particles (half-life = 14.29 days).

T-75 - flask - sterile plastic (capped) tissue culture flask, with a surface area for cell attachment of 75 cm^2.

T-25 - same as above, but with a surface area of 25 cm^2.

Trypsin - an enzyme, used at specified concentrations and selected incubation times and temperatures, to cause cells to detach from plastic surfaces of tissue culture flasks. Immediately after the cells are suspended, the trypsin is inactivated by the addition of medium containing serum.

* U. S. GOVERNMENT PRINTING OFFICE: 1991--561-052/40003